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Lyophilized liposome-based parenteral drug development: Reviewing complex product design strategies and current regulatory environments☆

Yuwei Wang [a,*], David W. Grainger [b,c]

[a] College of Pharmacy, California Health Science University, Clovis, CA 93612, USA
[b] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112, USA
[c] Department of Biomedical Engineering, University of Utah, Salt Lake City, UT 84112, USA

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ABSTRACT

Given the successful entry of several liposomal drug products into market, and some with decades of clinical efficacy, liposomal drug delivery systems have proven capabilities to overcome certain limitations of traditional drug delivery, especially for toxic and biologic drugs. This experience has helped promote new liposomal approaches to emerging drug classes and current therapeutic challenges. All approved liposomal dosage forms are parenteral formulations, a pathway demonstrating greatest safety and efficacy to date. Due to the intrinsic instability of aqueous liposomal dispersions, lyophilization is commonly applied as an important solution to improve liposomal drug stability, and facilitate transportation, storage and improve product shelf-life. While lyophilization is a mature pharmaceutical technology, liposome-specific lyophilization platforms must be developed using particular lyophilization experience and strategies. This review provides an overview of liposome formulation-specific lyophilization approaches for parenteral use, excipients used exclusively in liposomal parenteral products, lyophilized liposome formulation design and process development, long-term storage, and current regulatory guidance for liposome drug products. Readers should capture a comprehensive understanding of formulation and process variables and strategies for developing parenterally administered liposomal drugs.

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* Corresponding author.
E-mail address: yuwang@chsu.edu (Y. Wang).

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1. Introduction

Liposomes are sub-micron diameter spherical lipid vesicles comprising one or more biocompatible lipid bilayers with an aqueous core [1–3]. Liposome lipid bilayers create an internal aqueous solution core separated from the remaining bulk aqueous drug system, forming a compartment that permits two aqueous microenvironments to co-exist in the formulation. Encapsulated hydrophilic solutes dissolved within the vesicle core cannot readily pass through the intact liposome bilayer membrane. The bilayer comprises two phospholipid monolayers analogous in simple structure to a cell membrane barrier. The inner lipid layer faces the aqueous core while the outer layer faces the external bulk aqueous formulation. Due to curvature effects, packing geometries for phospholipids in these two layers are quite different for small (15–30 nm diameter) than larger liposomes. The inner monolayer leaflet curvature produces higher lipid packing constraints compared with the outer monolayer leaflet. This lipid bilayer composition has been extensively exploited, using natural, semi-synthetic and synthetic lipids and their mixtures to alter liposomal final biophysical properties to improve formulating, product stability, in vivo biodistribution, and therapeutic effects [4].

Due to the intrinsic amphiphilicity of their lipid bilayer shell(s), liposomes exhibit both lipophilic and hydrophilic properties that facilitate encapsulation of a broad range of pharmaceuticals. Since the advent of Doxil®, the first liposomal drug FDA-approved in 1995 [5], diverse liposomal drug carriers and formulations have been intensively investigated, patented and developed. Table 1 lists current commercial liposomal drug formulations used clinically and some specific attributes. This clinical formulary demonstrates the versatility of the liposomal formulation to deliver diverse types of therapeutics as aqueous nano-phase dispersions. Liposome formulations are extensively investigated for many different routes of administration, for example, parenteral, oral, transdermal, nasal, pulmonary and ophthalmic [6]. Nonetheless, the majority of liposome-based formulations in different phases of clinical assessments are intravenous or intramuscular injections [2,3,7].

Compared to conventional parenteral drug solutions, liposomal drug systems combining drugs into sub-micron lipid carriers as aqueous parenteral dispersions require additional considerations for regulatory approval, producing a more complicated development and regulatory process with higher costs and risks. Such aqueous formulated lipid dispersions exhibit known physical and chemical instabilities that both limit their widespread use, and often result in reduced product shelf-life [3,6,9]. This increases cost, quality control protocols and possible patient safety/efficacy and risk issues. Notable liposome drug product recalls have resulted [9,10]. During long-term storage, recognized formulation degradation problems for this drug class include phospholipid oxidation, liposome aggregation or fusion, lack of re-dispersibility, and drug leakage.

Lyophilization, also known as freeze drying, is frequently applied as a primary strategy to reduce known liposome formulation stability risks and to improve the shelf-life for liposome-based drugs [3,8,11]. Lyophilization is well-studied for various pharmaceutical formulations. Fig. 1 shows the classic phase diagram used to achieve freeze drying by phase transition known for aqueous systems under controlled conditions.

Commercial lyophilization methods for diverse drug formulations are well-developed [12–14]: over half of the >300 current FDA- and EMA- approved biopharmaceutical products are lyophilized, despite the high cost and long processing times required, supporting this approach as essential in producing reliable aqueous product stability for these biological drug products [15]. Analogously, lyophilization is also an accepted manufacturing approach for liposomes [8,11,16]. Lyophilization equipment and protocols are becoming sophisticated to permit careful control of drying equilibria and adherence to equations of state for reliably dessicating aqueous formulations and avoiding ice crystallization [15,17]. Process development and optimization for product prototyping typically uses laboratory-scale batch lyophilizers with small (0.1–0.5m^2) shelf areas. Pilot-scale batch lyophilizers (~5m^2 shelf areas) are then used to manufacture drug product for quality, stability and clinical testing requirements. This scaling facilitates other process optimization opportunities for de-risking drug product development. Post-production-scale batch freeze driers (shelf areas up to 50m^2) may be used for commercial product scaling [18]. In any case, limitations of batch lyophilization methods both for pilot product and process validation, as well as inconsistencies in using laboratory, pilot and production scale batch lyophilizers to qualify QbD and associated Critical Quality Attribute (CQA) product validation are recognized as intrinsic scaling risks [19,20].

Lyophilization at any scale is recognized to alter liposome physical structure, producing variability in formulation stability and reconstitution reliability [21]. Maintaining liposomal integrity during lyophilization is therefore a consistent challenge [21,22]. During freezing, lipid bilayers can rupture during ice crystal formation from either within the compartment or the external aqueous phase. Additionally, during drying, without sufficient protection, vesicles can irreversibly aggregate or fuse to a much larger lipid particles, producing drug leakage [21,22]. During aqueous reconstitution prior to administration, lipid bilayer integrity could be compromised as well due to lipid phase transitions induced by re-hydration. In addition, if incorporated drugs are proteins, peptides or genes, the challenges of maintaining vesicle and formulation stability are even greater. Given recent increasing focus on novel liposomal formulations of biologics [3,4], producing reliable protocols and formulation pathways for these evolving complex products is prudent.

To improve lyophilization methods and avoid these formulation compromises, many lyoprotectants, approved by FDA for pharmaceutical use, have been developed and used for marketed drugs [12,23]. The mechanism of lyoprotection has also been extensively investigated for decades [11,21]. This extensive history and experience has produced a very efficient and versatile design platform and evaluation system for developing parenterally administered liposomal lyophilized formulations. Below, formulation design strategies for liposomal lyophile drugs and process development approaches for liposomal formulation

Table 1
Currently approved clinical liposomal drug therapies [2,3,8].

Disease indication	Liposomal product name	Drug (API)	Lipid composition[a]	Company
Cancer therapy	Doxil®	Doxorubicin	HSPC, cholesterol and DSPE-PEG2000	Janssen Johnson&Johnson
	DaunoXome®	Daunorubicin	DSPC and cholesterol	Galen
	Mepact®	Mifamurtide	POPC, OOPS	Takeda Pharmaceutical
	Marqibo®	Vincristine	Egg sphingomyelin and cholesterol	Talon Therapeutics
	DepoCyt®	Cytosine, arabinoside	DOPC, DPPG, cholesterol and triolein	SkyPharma Inc.
	Myocet®	Doxorubicin	EPC, cholesterol	Elan Corporation USA
	Onivyde™	Irinotecan	DSPC, MPEG-2000:DSPE, cholesterol	Merrimack Pharmaceuticals
	Vyxeos®	Daunorubicin: cytarabine (molar ratio 1:5)	DSPC, DSPG, cholesterol	Jazz Pharmaceuticals
Viral vaccines	Epaxal®	Hepatitis A vaccine	DOPC, DOPE	Crucell, Berna Biotech
	Inflexal®V	Influenza vaccine	DOPC, DOPE	Crucell, Berna Biotech
Photodynamic therapy	Visudyne®	Verteporfin	PG (egg), DMPC	Novartis
	Visudin	Verteporphyrin	DMPC, PG	Novartis
Analgesics	DepoDur™	Morphine sulfate	DOPC, DPPG, cholesterol and triolein	SkyPharma Inc.
	Exparel®	Bupivacaine	DPPG, DEPC, cholesterol	Pacira Pharmaceuticals, Inc.
Fungal diseases	Abelcet®	Amphotericin B	DMPC, DMPG	Enzon
	Ambisome®	Amphotericin B	hSPC, DSPG, cholesterol	Astellas Pharma
	Amphotec®	Amphotericin B	Cholesteryl sulfate	Intermune
	Nyotran®	Nystatin	DMPC, DMPG	Aronex Pharmaceuticals
Acute hepatitis B, chronic hepatitis C	Lipoferon	Interferon-alpha 2b	Lecithin, cholesterol	Jardan
	Reaferon-es-lipint	Interferon-alpha 2b	Lecithin, cholesterol	Vektor-Medika

[a] PG (egg): egg phosphatidylglycerol; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; OOPS: 1,2-dioleoyl-sn-glycerol-sn-phosphatidylserine monosodium salt; DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE: distearoyl-sn-glycero-phosphoethanolamine; DSPG: distearoylphosphatidylglycerol; DPPG: 1,2-dipalmitoyl-sn-glycero-3 phospho-rac-(1-glycerol); DEPC: 1,2-dierucoylphosphatidylcholine; HSPC: hydrogenated soy phosphatidylcholine; POPC: palmitoyloleoylphosphatidylcholine; MPEG: methoxy polyethylene glycol; DMPC: dimyristoyl phosphatidylcholine; DMPG: dimyristoyl phosphatidylglycerol; DEPC: dierucoylphosphatidylcholine; DOPE:dioleoly-sn-glycero-phosphoethanolamine.

lyophilization are described. To date, a few publications have summarized liposome lyophilization methods and outcomes [11,21], but very few reports focus on issues specific to parenteral liposomal formulations that comprise many approved products and future desired formulations (e.g., for biologics). Important aspects of lyophilized liposomal aseptic formulations, covering lyo-liposomal formulation development, excipient selection and lyophilization process development, stability and storage considerations, and regulatory guidance and liposomal drug requirements for FDA approval are presented.

1.1. Liposome formulation development issues and challenges

The aqueous core compartment and biocompatible lipid layers endow liposomes with unique capabilities to encapsulate both lipophilic and hydrophilic drugs, creating a versatile delivery platform for both small and biomacromolecules, including DNA, siRNA, microRNA and proteins. Less robust biomolecules are protected as cargo within the carrier's aqueous center from degradation (i.e., from host circulating DNAses, RNAses, proteases), and from dilution in blood circulation before reaching target sites. Liposome outer lipid layers facilitate transport, cellular and tissue uptake, and intracellular processing. In addition, design and control of liposome intrinsic properties, such as particle size, charge, and lipid composition can produce changes in biodistribution to bias fractional dose delivery to certain organs or tissues [24,25]. This altered liposome biodistribution compared to free drug injection is responsible for much of the therapeutic benefit attributed to liposome formulations in the form of reduced dose toxicity and increased maximum tolerated dose [2,3,26].

With decades of development and clinical liposome drug delivery experience now recorded, some remaining issues and therapeutic challenges hinder liposome clinical performance. First, only small percentages of injected doses reach intended sites of therapy from systemic circulation [2,26]. While studies following labeled drug versus labeled lipid for liposomes delivered in vivo produce variable conclusions on biodistribution, most studies using covalent radiolabels on liposome components show <5% of injected dose to the target site as typical [27]. Liposome dose "targeting" using externally attached ligands (e.g., RGD peptides, antibodies, glycans) while widely claimed in models, rarely improves specific site targeting in humans, although lipid modifications can change biodistribution profiles [26]. Second, despite their common phospholipid shell composition, liposomes are not as immunologically inert as originally proposed. Many studies have now shown that some therapeutic liposomes trigger adverse immune responses, including complement activation, eliciting C activation-related pseudoallergy [28–31]. Repeated injection of PEGylated liposomes is also reported to produce accelerated blood clearance (ABC phenomenon) associated with enhanced liposomal systemic recognition and removal [32–35].

Physical and chemical liposome vehicle destabilization is another issue, especially when encapsulated drugs are nucleotides or proteins. Liposome fusion and aggregation is spontaneous with time in aqueous conditions. Therefore, low temperature transportation and storage (e.g., −20 °C to −80 °C) is always required, mandating careful and expensive cold chain custody for clinical product manufacture, distribution and storage. Taken together, translational progress of liposome drug delivery formulations to clinical use is very slow, tedious and expensive. Despite these disincentives, many preclinical studies showing therapeutic effects in vivo continue to motivate more clinical liposomal development [26]. While clinical performance is less definite or predictable, important clinical benefits are evident from select liposomal

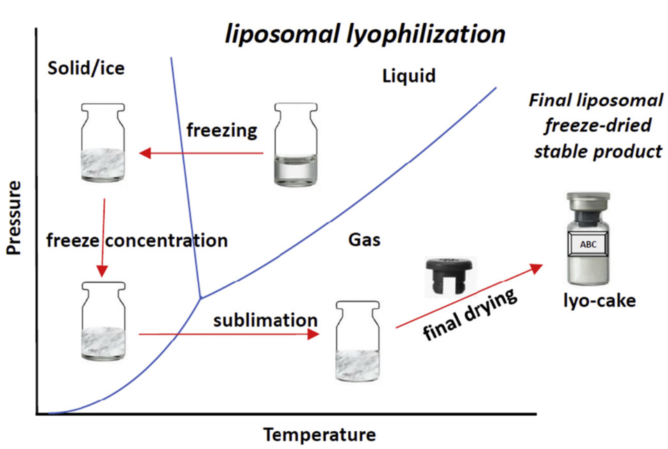

Fig. 1. Phase diagram of lyophilization phase transitions that couple heat and mass transfer.

approved drug products in certain indications that are generally compelling to new formulations.

1.2. Desired characteristics of a lyophilized liposome drug product

Liposomal instability remains a major translational limitation for developing liposomal formulations for clinic use. Narrow temperature controlled or cold chain product stability requirements during formulation or product storage are undesirable. Generally, liposome systems stored as aqueous dispersions at room temperature leak their encapsulated drugs; liposomal carriers aggregate and fuse also inducing liposomal drug release [21,22]. Hydrolysis and other aqueous phase chemical degradation reactions (i.e., lipid peroxidation) are always an issue with most any formulation [36]. Final product lyophilization is therefore commonly pursued as a validated approach to increase liposomal product shelf life and limit both physical and chemical product compromise. This approach is adapted from well-known lyophilization strategies used for unstable aqueous drug formulations for decades; liposome lyophilization has been studied routinely as an important strategy to stabilize diverse liposomal drug formulations [21,22,37,38].

Lyophilization uses carefully controlled freezing then subsequent drying (desiccation) conditions to remove water from the aqueous lipid dispersions, yielding a stable, largely dry powder formulation (i.e., lyo-cake). This dried liposomal product retains all non-volatile formulation components; formulation chemical composition is therefore known and stable based on formulating controls. Additionally, physical stability is equally important to drug formulation stability. This dry-state stabilization typically refers to reduced drug solid-state aggregation and degradation (e.g., hydrolysis), ready solid re-dispersibility upon aqueous reconstitution (no caking, aggregation or insolubility), and polymorph avoidance in the case of solid lyophilized injectable free drugs. Importantly, for liposomes, lipid vehicle structural maintenance stability in dry states under storage, and lipid bilayer reconstitution without drug leakage upon water addition prior to administration, are both critical features of lyophilization unique to this dosage form's stabilization [39].

Lyophilized drug product (e.g., lyo-product, lyo-cake) designs are used to facilitate liposomal storage in dry state forms that increase their shelf life and limit cold chain custodial demands [21,22]. Product release requirements for good lyo-cakes for liposomes are no different than for other drug lyo-products [40]. Desired characteristics for acceptable lyo-products include elegant cake appearance and short reconstitution times. In addition, reconstituted solutions and dispersions should preserve all the characteristics of the original formulation [40,41]. Specific to liposome formulations, particle size distribution, surface zeta potential, and drug encapsulation are essential dosage form evaluation criteria for dry cake product. Incorporated macromolecule drugs, including peptides, proteins and nucleic acids, in prospective new products must also retain native bioactive structures and conformations in reconstituted formulations.

2. Characteristics of liposome lyophilization

2.1. The lyophilization process and formulation destabilization issues

Lyophilization process technology is applied primarily to drug products that are unstable in aqueous solutions at room temperature for longer storage times. Protocols typically first create a frozen aqueous phase of the formulation, followed by a primary drying phase with water sublimation, then a secondary drying phase of water desorption. Proper control and monitoring of all three steps is mandatory to ensure drug product quality complies with its respective defined Critical Quality Attribute (CQA) limits in Quality-by-Design (QbD) formulation mandates (see Section 5 below) [19].

Liposome-based drug delivery systems, especially combined with biological drugs, rely on freeze drying as the only option to improve formulation stability and prolong shelf life both for vesicles and the active pharmaceutical ingredient (API). This is essential for liposomal drug translation to clinical use. Typical lyophilization processing for liposome formulations is highly similar, including freezing, primary drying and secondary drying to obtain the final dried liposomes with desired final moisture content specifications. However, for parenteral liposomal products, the lyo-cake rehydration/reconstitution step with shelf-life testing and storage considerations should also be counted as a critical final validation step for product acceptance (see Fig. 3). In addition, due to structural features unique to liposomes and critical to their delivery properties, the interior liposomal aqueous solution must be carefully considered separately for lyo-processing, in addition to standard bulk suspension considerations [42].

2.1.1. The freezing step [43]

As freeze drying and aqueous sublimation do not occur in the absence of bulk phase freezing and maintenance of its frozen state throughout sublimation, the aqueous formulation is cooled from room temperature and solidified under controlled conditions during this first lyophilization processing step. Water molecules form pure crystalline ice via homogeneous and heterogeneous nucleation, and separate from the rest of the aqueous components left in the liquid phase. Excipients present in the formulation complicate this aqueous phase separation during freezing (i.e., via freeze concentration). With increasing ice formation, the remaining liquid becomes more concentrated and viscous, and its freezing temperature is continually reduced by colligative influences (i.e., freezing point depression). This freeze concentration concentrates the formulation solutes as ice forms, and determine whether soluble excipients remain amorphous or crystallize in this concentrated milieu. At the end of this freezing step, the entire system is amorphous or crystalline solid, or has mixed co-existing solid phases of varying compositions and physical states.

Liposome suspensions during the freezing step also freeze concentrate, gradually separating from bulk aqueous solution solid phases, forming a frozen solids concentrate with the remaining phase separated formulation solutes while bulk water is freezing. However, the presence of the lipid vesicles with inner aqueous compartments and outer bulk extravesicular aqueous phase further complicates the freezing mechanisms, and stymies ready abilities to control it to stabilize liposomes during freeze drying [42]. Water molecules in bulk aqueous phases form ice crystals with decreasing temperature (−5 °C to −25 °C) [42], but internal vesicle compartments freeze differently, depending on their size and contents [44].

Significantly, resulting ice crystal size is important in balancing product drying rates (and cost) with potential liposome damage (product quality), and known to be influenced by bulk freezing rates carefully controlled through lyophilization processing technology. Quick-freezing leads to larger numbers of finer ice crystals and larger ice-water interface [45]. Small ice crystals produce highly ordered bulk structures with lower volume-surface areas, thus reducing their subsequent sublimation rate during drying steps [46,47]. However, small ice crystals normally generate less physical damage to liposome membranes, improving their integrity compared to large ice crystals. Larger ice crystals exhibit higher sublimation rates resulting in shorter formulation drying times. In the remaining concentrated liposome formulation bulk liquid, inter-liposome spacing becomes smaller during freezing and concentration, resulting in higher membrane densities so that phospholipid surfaces approach closely. This increases the compressive mechanical stress on the liposomal bilayer, inducing damage to the bilayer followed by leakage of encapsulated hydrophilic drugs [48].

With increasing bulk solution freezing, phase separation of bulk frozen water and increasing sequestered solute concentrations surrounding the liposomes produces an aqueous milieu distinct from the internal encapsulated drug aqueous phase. Therefore, a destabilizing osmotic stress is generated across the lipid membrane during freezing [42]. This building osmotic gradient can either shrink or swell the freezing liposomes, disrupting bilayer integrity and causing losses of internal liposomal solution as leakage [44], since internal solutions freeze at lower temperature (−25 °C to −45 °C) by homogeneous ice nucleation [42,49]. Compared with ice crystals formed in the external aqueous phase or on the exterior liposome surface, ice crystals formed internally in vesicles can produce serious damage to lipid membrane integrity [50–52]. Internal phase freezing also can phase separate encapsulated drug into new crystal polymorphs exhibiting variable dissolution rates upon drying or thaw [53–55]. Therefore, several freezing-related physical changes in liposome micro-environments potentially destabilize liposomes during this initial freezing step.

2.1.2. Drying steps

During the primary drying step, the initial lyophilizer vacuum is applied to the frozen product. Lyophilizer chamber pressures normally range from 50-200 mTorr, enabling ice sublimation at sub-ambient temperatures ranging from −30 °C to 10 °C [14,41]. Ice crystal morphologies during freezing (i.e., fine versus large) and drying conditions determine product drying times [56], a key parameter in manufacturing cost control. Products must be maintained in a solid frozen solid phase below their collapse temperature (Tc) [21] to minimize formulation solid physical structure changes, such as vesicle fusion or aggregation [57]. The Tc of amorphous solid systems is the same as their glass transition temperature (Tg), and is the eutectic temperature for solid crystalline systems [58,59]. Higher primary drying temperatures promote sublimation rates that result in shorter primary drying times [60]. The majority of water is removed during the primary drying step, requiring most (~80%) of the freeze drying process time (and hence majority of processing costs). Small amounts of residual water bound to polar solute molecules cannot be removed under primary drying conditions. The secondary drying step applies a similar vacuum but shelf temperature is increased to facilitate water desorption for the final desired residual moisture of the product [61,62].

Currently, methods to accurately predict appropriate residual water levels for a given formulation do not exist: physical assessments and analysis of each formulation with different residual water contents are required. Methods to prepare dry product samples with different water contents for such studies require a mechanistic understanding of the highly variable physical properties for samples prepared with different methods and residual water. Variables including the extent of structural relaxation in the formulation solid dry glassy state, the apparent acidity of the dried product, and the resulting liposomal structure in the lyo-cake must be considered. Knowledge of the phase composition of the final freeze-dried lyo-cake, including fractional crystallinity of the excipients, and possible changes in such crystallinity during stability testing and storage is important to understanding residual water content and resulting product stability [63].

Analogous to freezing, drying steps can therefore also destabilize liposomes in diverse ways. If the temperature rises above the collapse temperature during primary drying, the developing product cake can collapse. If liposomes completely aggregate or fuse, a liposome suspension will not be obtained after rehydration. The optimal temperature for secondary drying also needs to be investigated and then carefully controlled in processing. If too high, overheating during drying can induce liposome collapse and destabilization. In addition, the stability of the encapsulated active (API) under secondary drying temperatures must be carefully considered, especially for biopharmaceuticals.

A summary of the primary lyophilization process steps for time, temperature and pressure is shown in Fig. 2.

Recently, algorithms for determining the design space for product drying steps have become available [64], and mathematical models to address lyophilization scaling and the multi-variable complexities in process optimization are published [65]. Drying process optimization for lyophilization can be monitored in situ in real time for liposome formulations as well [66]. These developments should improve approaches to design space controls and QbD aspects of lyophilization.

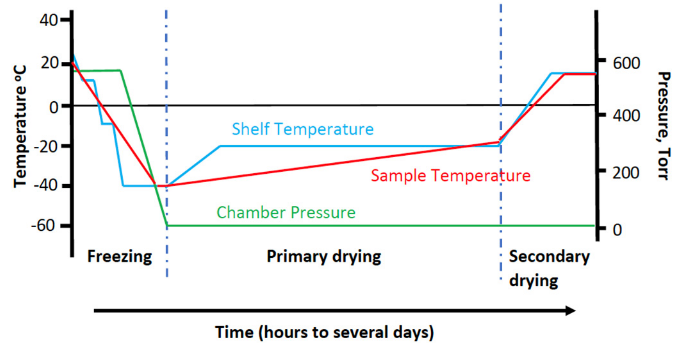

Fig. 2. Schematic of a typical pharmaceutical product lyophilization cycle: freezing, primary drying and secondary drying phases over time under temperature and pressure.

2.1.3. Rehydration or reconstitution steps

Rehydration or product aqueous reconstitution as required for formulation parenteral delivery to patients after storage is normally not included in the freeze-drying process validation. However, this is a very important step for validating liposome formulation stability and product utility. A good-looking final product dry powder or lyo-cake may not preserve vesicle drug encapsulation or required particle size distribution upon rehydration [39]. If any of these characteristics change, drug product efficacy is also changed, meaning that all pharmacokinetic and pharmacodynamic product data based on fresh drug suspension prior to freeze-drying would need to be re-established. Selection of appropriate reconstitution media to generate a final formulation solution with desired tonicity must therefore be carefully considered from early product development stages.

In addition, short-term stability of the reconstituted aqueous liposomal dispersion at room temperature must also be validated to accommodate clinical use. That is, the final re-dispersed product must remain stable for the duration of parenteral administration (i.e., injection or infusion), or under further dilution or mixing with other bulk infusion solutions. Structural recovery of liposomes after reconstitution is distinct from other lyo-cake reconstitution criteria, and critical to evaluate the lyo-process for this formulation. Therefore, the endpoint for successful liposome formulation lyophilization, distinct from small molecule lyophilized drugs, should best include the reconstitution step and administration steps, beyond a suitable lyophilized cake product and shelf-life testing.

2.2. Mechanisms of lyoprotection for liposome formulations

Given the array of possible destabilizing effects under lyophilization processing for liposome systems, liposomes cannot reliably survive freezing damage and dehydration destabilization [21,22,42]. To address this need, lyoprotectants are used as liposome-specific excipients [21,67]. Certainly, lyoprotectants are not a new addition for improving freeze-drying processes for liposomes. Although many diverse chemistries and compositions are reported for small molecules [12,13], lyoprotectants used in liposome formulations are largely focused on only a few sugars: monosaccharides and disaccharides [42,68–71]. Many hypotheses for lyoprotective mechanisms – both physical chemical and structural - have also been proposed [21,67,71–73].

The water replacement hypothesis [74] proposes that non-volatile lyoprotectant molecules replace water molecules by forming stable hydrogen bonds with liposome lipids at the bilayer surface without altering lipid bilayer structure as water is removed. Upon dehydration, water removal reduces lipid head group alterations, retaining tighter packing of the lipid layers, and favoring increased close-range stabilizing van der Waals interactions between lipid chains. Lyoprotectants, typically sugars and polyols (i.e., disaccharides) are added to the freezing milieu to replace water in stable hydrogen bonding with formulation components. With lyoprotectants, spacing between lipid head groups can be maintained and hydrophobic interactions between acyl chains reduced during drying steps to retain sealed membrane structures during freeze-drying [75]. Commonly used lyoprotectant, trehalose, is an example [76–78]. As a hydrated polar sugar with extensive hydrogen bond capabilities, trehalose hydrogen bonding with phospholipids is enhanced upon dehydration compared with its hydrated state [68].

The vitrification model [74] proposes that lyoprotectants form a high viscosity aqueous phase with liposomes that protects spacing between adjacent lipid bilayers, avoiding fusion and mechanical disruption from membrane conformational changes during freeze drying [44,48,79]. These formed glassy phases are thought to reduce the surface tension of lipid bilayers by interacting directly with the liposome surface as water is removed [80]. Studies have shown that these two mechanisms do not contradict to each other, but can co-exist as required for liposome integrity protection under freeze drying [21,81].

2.3. Lyo-formulation design assessment

For liposome drug lyophilization, several indicators help evaluate and quality control the resulting lyo-products. The most important parameters supporting liposome protection during the freeze-drying process are: reconstituted particle size, zeta potential and drug encapsulation efficiency of the reconstituted liposome dispersion. Diverse process analytical technologies (PAT) are typically applied during product assessment and validation processes to validate product attributes under design control and during produce life cycle management. Particle size and zeta potential influence liposome tissue targeting, recognition and elimination, opsonization and immunogenicity in the body [26]. Particle size changes can change the injected product biodistribution, pharmacokinetic and pharmacodynamics profile. However, while maintenance of particle size and zeta potential cannot guarantee product biological activity, they serve as prerequisite indicators both accessible and convenient for monitoring, and correlated to preserving liposomal efficacy [82]. Drug encapsulation efficiency represents the drug encapsulated fraction retained within the liposomes in a reconstituted dispersion vs. the total drug amount contained in the same reconstituted liposome dispersion. After lyophilization, total drug in the product should not change, but the drug percentage within liposomes may change. Therefore, assessing final encapsulated drug amount partitioned between internal and external carrier phases is critical for liposomal drugs. This is another requirement distinct to liposome lyophilization product profiling compared to other parenteral drugs.

2.4. Challenges of lyophilizing liposomes with biological drug payloads

Destabilizing challenges to liposomes undergoing lyophilization at each freeze-drying step are substantial in producing reliable and validated products for clinical use. Beyond this general quality control feature, the fate of encapsulated drug payloads must be considered in liposome lyophilization benchmarks. Compared with small molecule liposomal products, reconstitution strategies and short-term stabilities of reconstituted dosage forms comprising encapsulated biological agents (e.g., therapeutic peptides, proteins and nucleic acids DNA and RNA) must be considered differently. Increasing interest in liposomes as a favored delivery carrier for improving cell therapy, for delivery of various growth factors or siRNAs produces the presumption that encapsulated formulations of these molecules are reliably stable [83–87]. The first recently FDA-approved RNAi therapeutic, ONPATTRO™ (Patisiran, Alnylam Pharmaceuticals, USA) is a non-liposomal lipid nanoparticle-encapsulated formulation. Alnylam's ALN-VSP for systemically delivering siRNAs to treat liver cancer, currently in clinical trials, is also a lipid nanoparticle-based formulation [88]. Several ongoing clinical trials of injected liposome-based siRNA therapeutics are also known [89]. These recent parenteral lipid-encapsulated siRNA dosage forms, as well as recent clinical trials involving liposomal vaccine particles, enzymes and lipopeptides [3], indicate that formulations of liposomal biologics are of increasing interest. Stability issues should figure prominently in their designs and successful final formulations.

When the encapsulated active is a biological drug, the stability issues from desired room temperature storage are very likely to drive lyo-product formulation to facilitate ease and reliability of liposomal product handling, delivery and storage. In these cases, methods to best preserve the bioactivities of the encapsulated drug during freeze drying processing steps are an additional and substantial challenge. Lyo-protection mechanisms for liposomes are based primarily on their interactions with lipid bilayers; the same mechanism applies also to protein drug lyo-protection as well. Lyoprotectant molecules directly bind to proteins to preserve their structure under dehydration and eliminate physical stresses encountered during the drying process [90]. Studies also showed that siRNA liposomes and lipoplexes both lose significant activity after lyophilization in the absence of lyoprotectant [67]. Therefore, lyoprotectant selection for liposomes carrying biological drugs must consider interactions of lyoprotectants with both membrane lipids and encapsulated bio-actives (APIs) in freeze concentrations and resulting drying processes.

3. Formulation requirements and designs for parenteral lyophilized liposome drugs

The complete formulation composition (e.g., API, buffer, bulking agents, stabilizers, surfactants, lyoprotectants and other excipients) all affect the freeze-dried cake stability and quality of the reconstituted product, as well as determining the freeze-drying processing temperature, and therefore the cycle time, and processing costs. A comprehensive product specification inventory or QbD analysis considers each of these variables. Fig. 3 shows the comprehensive parameter set associated with a typical liposome formulation matrix undergoing lyophilization.

3.1. Excipient selection for liposome lyophilization

Excipients are ingredients in pharmaceutical formulations other than the active drug in the finished pharmaceutical dosage form. Excipients are an evolving, extremely diverse chemical and physical set of thousands of compounds added to drug formulations for specific functions. Importantly, for lyophilization purposes, excipients exist in different physical states that may interact differently with liposomes. Amorphous excipients form a glassy matrix below the glass transition temperature and can associate with liposome surfaces, lipids and APIs, providing protection (i.e., lyoprotection), while crystalline excipients form a phase separated, ordered crystal structure upon freezing, providing structural and mechanical support for the generation of an acceptable lyophilized cake. Some excipients can produce both an amorphous and a crystalline structure, depending on lyophilization conditions. Formulations often contain both amorphous and crystalline excipients intended to provide optimal physical and biochemical product stability. Therefore, lyophilization processes must be properly designed to consider the critical temperatures and freezing kinetics associated with both the amorphous and crystalline phases formed during the freezing and drying processing, and which forms provide ultimate formulation stability.

Excipients shown capable of stabilizing products during liposome lyophilization processing and storage, and approved by regulatory agencies for parenteral formulations are highly limited. The FDA regulatory requirements for parenteral formulations are quite strict: they must be proven safe, non-toxic, sterile, pyrogen-free, and particle-free. From a therapeutics development perspective, selecting an excipient for freeze-drying processing will not emphasize innovation, but instead precedent and safety. Normally at this development step, liposomes suspension formulation designs are already completed in order to

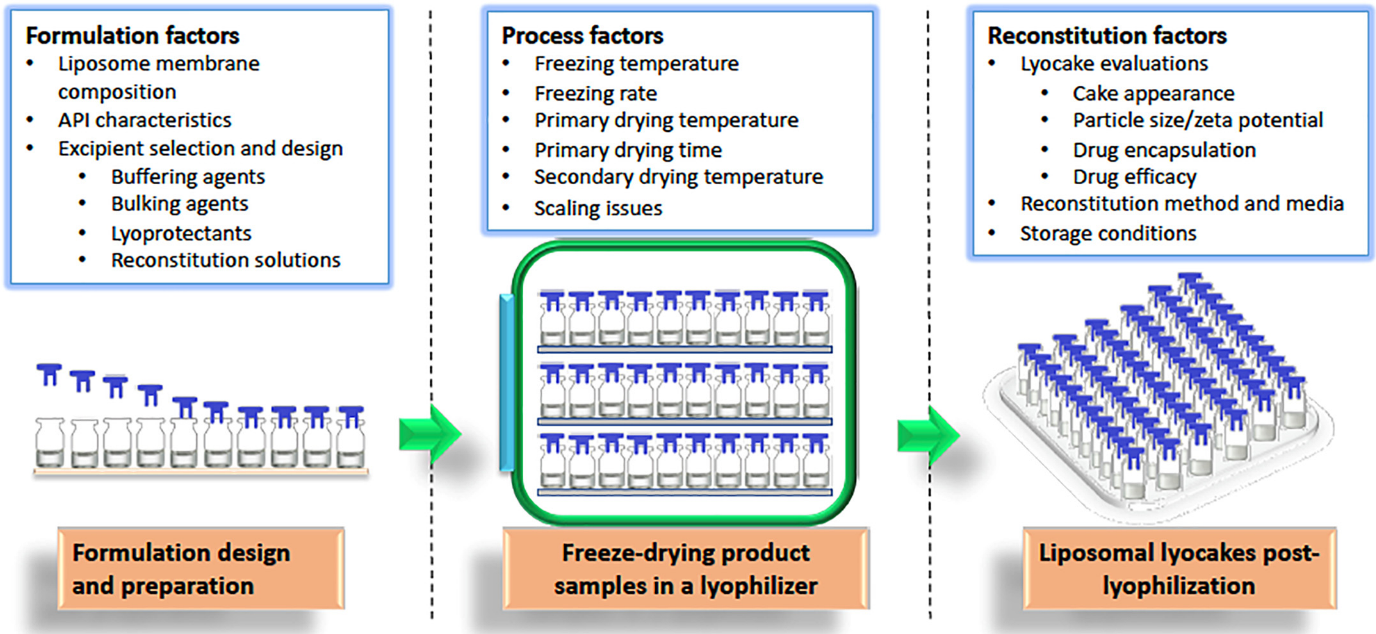

Fig. 3. Overview of the liposome formulation matrix for lyophilization and reconstitution required for stability and shelf-life stabilization in pharmaceutical product development.

facilitate and expedite FDA review steps. Therefore, early selection of FDA-approved excipients is a priority for all drug developers. Novel excipients must be available in pharmaceutical grade, and pass regulatory requirements similar to drugs, such as toxicology, ADME, pharmacokinetic and carcinogenic studies, prior to approval of the formulation [13]. Any new components in the liposome formulation not currently listed in the FDA's Inactive Ingredients Database or outside the limits provided in the Database require extensive safety justification, similar to any non-liposomal inactive ingredients. Obtaining this information is costly and delays development. Avoidance of these costly and time-consuming validation steps emphasizes a non-innovative, standard excipient selection common to liposomal formulating.

3.1.1. Buffering agents

Because of pH influences encountered during freeze-drying, both liposomes and biological drugs need constant pH value stabilization in the system during freeze drying, and reconstitution and storage. Hence, buffering agents are added into the formulation. Before selecting a buffer, a stability profile of the specific drug system with pH should be known. A buffer system capable of covering the pH of maximum stability of drug is then selected [41]. Among the commonly FDA-approved buffer systems for injectable drug products, phosphate/succinate/tartrate buffers should be avoided due to known pH shifts [91]. Sodium and potassium phosphate salts crystallize during freezing, removing their buffering capacity and leading to drastic pH shifts (e.g., 4 units lower) [92]. Acetate buffer can be partially lost during drying due to its volatile nature [23]. Citrate buffer can be used for lyophilization if the drug system's stable pH range is within 3.0–6.2, since citrate does not crystallize during freeze drying but remains amorphous [23]. Tris and histidine buffers are examples of pharmaceutically acceptable solutions [41]. HEPES buffer has been favored for liposome preparation because it offers more protection against lipid peroxidation compared to Tris buffer, and is also used for FDA-approved injectable drugs [93,94].

The selected buffer concentration should be set as the lowest required to reliably maintain the formulation pH in order to minimize amounts of inactive ingredient components in the liposome formulation [41].

3.1.2. Bulking agents

Analogous to their use in small molecule lyophilized formulations, bulking agents are the major component of liposomal lyo-cakes and necessary to provide requisite cake physical structure to facilitate liposome dispersion during freeze drying. The cake structure is highly porous to improve water escape and evaporation from the product during the drying process. Commonly used bulking agents including mannitol, glycine, glucose, sucrose, lactose, trehalose and dextran. However, crystalline bulking agents are not suited to liposomes formulations since solid crystallization during freezing can damage liposome membrane integrity, specifically attributed to mannitol, glycine, and glucose [95–97]. Trehalose is reported to crystallize during annealing at −18 °C but dehydrates to an amorphous anhydrate in the final dried product [78]. Therefore simply analyzing the final product may not be sufficient to reveal whether lyoprotectants crystallize during the entire freeze drying process. For PEGylated liposomes, dextrans failed to prevent aggregation during freeze drying and storage, most likely attributed to the mixing incompatibility between dextrans and PEG [72]. Phase separation is reported for mixing concentrated dextran solutions with concentrated PEG solutions [98], increasing with dextran molecular weight [72]. Attention must also be directed to those agents having reducing groups, such as glucose and lactose, that may change the zeta potential of dried liposomes.

If two different sugars are judged to both be suitable for product lyophilization, then the sugar with the higher glass transition temperature (Tg) should be selected. Based on the Gordon-Taylor equation (Eq. 1), [99] the resulting Tg of the mixture will be higher if the components' Tg values are higher:

$$Tg(mix) = \frac{W_a Tg_a + k(1-W_a)Tg_b}{W_a + k(1-W_a)} \quad (1)$$

where Tg_a and Tg_b are glass transition temperatures of components a and b, respectively. W_a is the weight ratio of component a, k is a constant representing the ratio of the thermal expansion coefficient differences between component a glassy state and liquid state, and also that for component b. A significant advantage of the higher Tg component in a formulation is improved stability during drying and storage.

3.1.3. Lyoprotectants or stabilizers

In addition to bulking agents, lyoprotectants play an important and distinguishing role in protecting liposome structural integrity during freeze drying and subsequent storage. Two proposed mechanisms for stabilizing liposomes during lyophilization are explained in Section 2.2 (*vida infra*). Monosaccharides are too small and unable to stabilize liposome dry powders due to their low Tg [100], so they have not been as effective as disaccharides in this role [101]. Disaccharides, such as sucrose and trehalose, have been proven to be most effective in protecting liposomes and protein actives during lyophilization, storage and rehydration [102]. They exhibit reduced hygroscopicity, low chemical reactivity and higher glass transition temperature of the maximally freeze-concentrated fraction (Tg') [70]. Sugars inhibited fusion of phospholipid membranes in the liposome dehydrated state and preserved membrane lipid distributions similar to the hydrated state [69,76]. After adding sugars to dispersed liposomal systems, sugar molecules form hydrogen bonds with phospholipids, so the membrane gel-to-liquid crystalline phase transition temperature (Tm) is reduced due to less tightly packed phospholipids. This permits the liposomal phospholipid membrane to retain a single phase during both drying and rehydration processes, avoiding structural transitions and drug leakage [68,103,104]. Oligosaccharides, such as inulin, were also shown to provide the same protection to gene delivery-based liposomes during freeze drying compared with disaccharides, suggesting that oligosaccharides should be better than disaccharides due to their higher Tg and reduced crystallization tendency, improving stability for storage purposes [72,105].

Hydroxypropyl-β-cyclodextrin (HPβCD), a cyclic oligosaccharide formed of glucopyranose units, has been approved for used in parenterally administered drug formulations [106]. HPβCD has also been proven to stabilize liposomes during freeze drying [107–109] with a high Tg' (−8 °C) which potentially increases the Tg' of the lyophilized mixture and increases primary drying temperature. HPβCD has exhibited excellent stabilization of PEGylated liposomes in both spray- and freeze-drying processing. The dry product can be stored at room temperature with no changes in size distribution of the reconstituted liposome dispersion [109]. The large number of hydrogen donors and acceptors in HPβCD may be the mechanism in the efficient replacement of water molecules on liposome membrane surfaces during freeze drying process, providing protection [107,109]. Therefore, HPβCD is now validated as an excellent membrane lyoprotectant for parenteral liposomal formulations. Lyoprotectant amounts should be controlled to the minimum to provide sufficient formulation protection.

3.2. Reconstitution solutions

Lyophilized parenteral products must be reconstituted with sterile diluent before administration, usually 5% dextrose solution, normal saline, bacteriostatic water, or sterile water for injection. Infusion products need further dilution for parenteral application [13]. Dosage forms for parenteral administration should be isotonic with human plasma to support blood and tissue compatibility and avoid adverse effects. Clinical water for injection (WFI) is recommended by the US FDA for reconstitution. Bacteriostatic Water For Injection should be avoided due to potential associated toxicity risks [110]. Custom, specific reconstitution solutions required for use should be provided in the drug package, increasing costs and inconvenience for storage, and risks for improper patient delivery. The composition of soluble components in liposome dispersions ready for lyophilization must be precisely formulated to ensure that the final diluted product ready for administration is isotonic with the site of administration. The typically ideal tonicity for parenteral formulations is 275–295 mOsm/kg [13]. Reconstitution time should be rapid and reliably consistent in the clinical user's hands: ideally, products should rehydrate and spontaneously disperse immediately after adding reconstitution solution. If a lyo-cake is not completely dissolved and dispersed, the undissolved fraction would need to be filtered prior to administration, resulting in loss of dose as filtered particulate matter.

3.3. Formulation design for lyophilized liposome formulations

Several parameters and components typical to most pharmaceutical lyophilized formulations, and some relevant to lyophilized liposomal formulation quality control and dosage form integrity, have been described (*vida infra*). More specific liposomal design considerations for lyophilized dosage forms for parenteral administration follow here.

3.3.1. Liposome membrane composition as a critical formulation design requirement for lyophilization and reconstitution

Lipid membrane compositions described for liposome lyophilization and delivery stability are diverse, highly proprietary in commercial formulations and supported by variable levels and quality of published data (e.g., see Table 1). Generally, membrane lipids comprise a majority of naturally derived phosphatidyl- ethanolamines and cholines as mixtures with other natural and synthetic lipids, surfactants and cholesterol. Cholesterol commonly erases a lipid membrane phase transition in water and its presence in liposomes is reported to reduce encapsulated drug leakage during rehydration of liposomal dry cake [111]. Cholesterol can also reduce fast cooling-induced damage to lipid bilayers by increasing the stability of the bilayers' fluid phase [112]. Cholesterol is also reported to reliably form hydrogen bonds with lipid polar heads in dry liposomes, reducing their Tm after dehydration [113]. In addition, cholesterol presumably facilitates the interactions between sugar lyoprotectants and lipid head groups by increasing lipid intermolecular spacing [114].

As increased circulation time is often desired for parenteral liposomes to improve tissue uptake from blood, using lipids conjugated on their head groups with inert hydrophilic polymer, polyethylene glycol (PEG, generally regarded as safe, “GRAS“) to decorate the liposome surface is common. Doxil® is a precedent PEGylated liposome anti-tumor doxorubicin product exhibiting prolonged circulation and a reduced volume of distribution, reducing known cardiotoxicity for the free drug, doxorubicin [5]. PEG-grafted liposomes are often referred to as “stealth liposomes” [1]. PEG surface presentation (PEGylation, [26]) is often highly hydrated, and shown to reduce adsorption of various blood proteins, reduce complement activation and increase liposome circulation times [26]. PEG is biocompatible in many applications, soluble in both aqueous and organic phases, and an FDA-approved excipient for pharmaceutical use. Incorporating PEG into liposome formulations improves membrane stability and overall performance of the dispersion [4]. Compared with non-PEGylated liposomes, PEGylated liposomes exhibit lower rates of aggregation when the dispersion freezes above the Tg', which indicates effects of stable PEG polymer hydration and steric repulsion on PEGylated liposome surfaces [72]. Surface-coated PEG is speculated to contribute synergistically to inhibiting ice crystal formation during freezing by controlling the vesicle's hydration layer formation to reduce ice nucleation [100]. However, these liposome bilayer modifications are not sufficiently strong to protect liposomes during the freeze drying process. Despite PEG surface modifications, liposome dispersions still require added lyoprotectants for freeze drying to avoid aggregation or fusion [115].

3.3.2. Lyoprotectant design for parenteral liposome dispersions

3.3.2.1. Different lyoprotectant compositions are used for aqueous core and bulk systems. It has been suggested that it is essential to distribute sugars on both sides of the liposomal bilayers for effective lyo-protection compared with only on one side [77]. Adding trehalose to both sides of liposome membranes demonstrates prevention of both freeze-induced dehydration and inner solute leakage [42]. Most studies have applied identical lyoprotectant concentrations to both sides of the bilayers for liposome lyophilization; few report different or asymmetric sugar distributions on the two sides. One study reported only small amounts of trehalose needed for liposome interiors for best protection, and adding more into the liposomes had no further effect on stability [116].

However, different trehalose protection effects were reported opposing leaflet sides of DPPC liposomes, suggesting higher inner and lower outer trehalose concentrations that provide better membrane stabilizing effects [68]. Specifically, this study showed increasing exterior trehalose concentrations to decrease membrane Tm. Therefore, precise lyoprotection effects attributed to differential distributions of lyoprotectants across barrier bilayers vary among different formulations, and trends in their asymmetric formulation in liposomes lack consensus.

3.3.2.2. Strategies for combining more than one lyoprotectant. When multiple stability needs must be met, combining two or more protectants is necessary. When the total solids formulation content is <2%, bulking agents are needed to provide solid structure to the lyo-cake [23]. For liposomes, lyoprotectants like monosaccharides become more effective when added simultaneously with other excipients for stabilizing liposome bilayer micro-structures during lyophilization and storage [101,117]. In other situations, allowing lyoprotectants to increase a formulation Tg while simultaneously providing direct interactions with lipid bilayers requires a strategy for combining protectants into a single formulation. Mixtures of phosphate anion and sucrose were shown to improve liposomal solute retention to 85% compared with complete leakage using phosphate alone, and 75% retention with sucrose alone with egg phosphatidylcholine liposomes after freeze drying. The excipient mixture dramatically increased the formulation Tg by hydrogen bonding networks between phosphates and sugars [117]. Combining glucose and hydroxyethyl starch was reported to stabilize liposomes, but alone, glucose cannot inhibit liposome fusion, and hydroxyethyl starch cannot stop leakage of entrapped solute during drying [101]. Mannitol alone does not provide much protection due to its crystallization during freeze drying, but after combining it with glucose, the resulting liposome drug retention reached 86.5% [118]. Mannitol combined with trehalose also was proven to provide good protection to liposome dispersions. A lyoprotectant composition combining a saccharide and cyclodextrin for lyophilized liposomes is also disclosed in some patents [119].

Multiple excipients used in formulations also produce potential adverse effects; this should be evaluated. Since sugar lyoprotectant use is common for liposome drugs, patients could suffer from transient high blood sugar levels during infusions or following liposome administrations. Sugar content in the administered dose should be clearly provided on product labels and its effects monitored after administration, especially for diabetic patients. Formulations with reduced sugar excipient amounts, alternative lyoprotectant or bulking excipients or co-administering another drug to control blood sugar levels should be designed in new liposome formulation development.

4. Liposome lyophilization process development

4.1. Lyo-cycle development for liposomal formulations

As the process of liposome lyophilization with the three lyo-cycle steps - freezing, primary drying, and secondary drying - should achieve the desired quality of final product with the least cost and highest quality control, each freeze-drying stage must be optimized and well controlled. Beyond formulation compositional considerations (e.g., excipients, API, lipids) the technical impact factors in each processing step are also important to evaluate using validated PAT methods. Given the current focus on parenteral lyophilized products, the majority of these products are manufactured using freeze drying, either in vials or pre-filled syringes [14]. Thus, relevant process parameters in this case are based on controlled freeze drying using pharmaceutical lyophilizer instrumentation. From a sterility assurance point of view, lyophilization is a final handling step for many parenteral drug products. Since terminal thermal sterilization is not applicable to liposomal drugs, it is essential to control the entire process under sterile conditions (aseptic processing) to ensure aseptic final product quality.

4.1.1. The freezing step

Several process parameters in the freezing step determine ice nucleation, impacting the following drying rates and structures of the final dried lyo-cake that influence stability and drying. These parameters include freezing temperatures, cooling rates, and holding time. Freezing converts aqueous liquid formulations into solid states. For liposome dispersions, the end point temperature for these formulations should be below the mixture's Tg'. For products in vials, lyophilizer shelf temperature is normally 2 °C lower than this Tg'. To ensure that all vials in different lyophilizer shelf locations reach the final desired temperature, the final freezing step temperature is normally held constant for one hour if the filling depth of vials is less than or equal to 1 cm. Longer holding time is needed if filling depth is greater, which is generally not favored due to increased freezing and drying times, and therefore increased cost [14].

Adding a specified post-freezing annealing step to liposome freezing protocols could be considered to increase drying rates and reduce primary drying times. Post-freeze annealing times above Tg' could increase drying rates [21]. Cake produced after annealing showed larger pores that improve water vapor sublimation during primary drying [120]. Annealing facilitates completion of crystallization, such as unfrozen water in the amorphous matrix. In addition, small crystals may further grow into larger ones, reducing sample heterogeneity and increasing sublimation rates during primary drying [45,121]. Annealing effects on liposome lyophilization showed that adding annealing steps significantly improved cake appearance, reduced primary drying time by 15% compared with conditions without annealing, and improved encapsulated solute retention with annealing [122]. Due to limited data supporting annealing to date, general rules governing annealing effects on liposomal lyophilization do not exist. This may also depend on excipients, lipid composition and particle size.

When using pharmaceutical-grade programmable freeze drying units, maximum cooling rates are normally 2 °C/min. Rates of 1 °C/min have been suggested as a good starting point for optimizing lyo-cycles to obtain uniform ice structure, either in each vial or among all vials [14]. Towards the same purpose of improving crystallization homogeneity, holding products at 5 °C for 15–30 min followed by −5 °C for 15–30 min is recommended as well [14].

4.1.2. The primary drying step

During primary drying, all free ice crystals in lyophilized products should be sublimed. Given water's substantial specific heat and high boiling point, this drying step is basically a heat transfer exercise to maximize both energy and mass transfer efficiency and homogeneity during lyophilization. The primary drying step normally is the longest step during a freeze-drying cycle, so optimizing it for minimal duration has significant economic impact on formulation processing. Chamber pressure in the range of 100–150 mTorr provides optimal homogeneity for heat transfer [14]. The higher the primary drying temperature, the shorter the primary drying time; a 1 °C increase in drying temperature can decrease primary drying duration by about 13% [123]. Conventionally, the primary temperature should be set 5–10 °C below the formulation Tc or Tg'[21]. Liposomal lyo-formulations have very low Tg' values due to their added lyoprotectants such as sucrose (Tg' ~ −34 °C) or trehalose (Tg' ~ −29 °C) [12,23]. Therefore, by following conventional rules, the primary drying time will be very long, hence costly. However, much work has shown that lyophilized liposomal dispersions can be primary dried at temperatures much higher than their Tg'. siRNA-containing DOTAP:DOPE liposomes (size = ~100 ± 75 nm) were dried at 28 °C with glucose (Tg' −43 °C), sucrose, trehalose and lactose (Tg'

−28 °C) separately. The resulting liposomal product transfection efficiency with four lyoprotectants did not significantly change compared to freshly prepared liposomes, despite the drying temperature greatly above the Tg' values [16].

Fluconazole multilamellar liposomes with the trehalose concentration above 3 g/g lipid retained up to 93% fluconazole loading after drying at −10 °C [124]. Another QbD study also showed that pravastatin-loaded liposome dispersions with trehalose at molar ratios 8:1 to lipid can be dried at −20 °C and preserve particle size, encapsulation efficiency and acceptable cake appearance with annealing steps involved [122]. These phenomena were observed for protein formulation lyophilization processing as well. Studies report that some protein formulations can be dried at temperatures above their Tg' without compromising protein bioactivity [125,126]. Analysis suggested that protein unfolding time was insufficient, meaning that if protein structural unfolding is slow enough versus the processing time scale, formulation drying is complete before significant protein structural changes affecting bioactivity can occur [14]. This mechanism for proteins may also be applicable to liposomes: when drying at a temperature higher than Tg', the increased sublimation rate is fast enough to complete product drying before significant bilayer structural changes.

Formulation vials complete their primary drying at different times depending on shelf placement. Due to additional radiative heat from lyophilization chamber walls and doors, vials at the shelf edges dry faster than those in the shelf center. This has been previously described as an important issue for freeze dry process scaling, and is addressed using mathematical models and a multi-parametric approach [65]. Thermocouples are most commonly used to monitor primary drying temperatures and endpoints. They can be placed in vials in representative locations (e.g., front, back and center shelf locations). Thermocouple-containing vials always dry faster than the non-containing vials due to lower product hydration resistance. Therefore, an additional drying period of 10–30% is often added for security [127].

4.1.3. The secondary drying step

The purpose of the secondary drying is removal of absorbed water. After primary drying, especially for non-crystalline solid products, like liposomes, 5–20% residual water still remains after the primary drying step [14]. Generally speaking, the desired final residual moisture level for formulation stability is <1% [128], and for pharmaceutical liposomes, moisture contents of 1% or less are suggested [122]. It is well known that the higher the residual water content, the lower the product stability, and that this also adversely affects subsequent reconstitution properties [22]. Such water reduces the product Tg, increasing risks of producing a Tg below the storage temperature [122]. When product storage temperature is higher than Tg, amorphous liposome components cannot remain in a glass matrix [11]. Liposome storage stability can be reduced by high residual water content, due to lyoprotectant crystallization [129]. The optimal storage temperature has been proposed to be 50 °C below Tg [11].

No significant physical instability or chemical degradation was reported in doxorubicin-containing liposomal cakes with <1% residual water after storage for 6 months at temperatures up to 30 °C [130]. However, for proteins, <1% residual moisture results in loss of protein bioactivity after reconstitution [21]. For protein-containing liposomes, removal of the protein's hydration shell is the major stress during drying, resulting in loss of protein function [131]. Previous studies also suggested that the inner liposome monolayer is partially dehydrated compared the outer layer [68]. Maintaining local availability of water inside the liposome core for encapsulated protein drugs or complex biological products is therefore necessary. A liposome core surface could therefore be deficient in optimal residual moisture levels, requiring that higher residual formulation water levels be maintained above than the general formulation requirements.

During the secondary drying step, shorter run times at high shelf temperature are preferred because water desorption rates decrease significantly with time at a given temperature [14]. The duration of secondary drying time can be determined by measuring product residual moisture at different drying time points. This process can be much simplified by using a "sample thief": equipment installed on the door of freeze-drying instrumentation to product a separate vacuum space for transferring samples from chamber shelves to outside the instrument for analysis.

4.2. Rehydration/reconstitution process design for liposomal lyocakes

Most freeze-drying studies of liposomal drugs have stopped at obtaining an acceptable cake and characterizing the reconstitution solution as endpoints. However, further stability studies of the reconstituted liposomal products mixed with diluent under in-use conditions of storage and clinical use are recommended by the FDA. Studies including physical, chemical and microbiological tests, and in-use or storage intervals, durations of admixed or unused liposome product that no longer pass product specifications and must be discarded all should be determined [132]. Information regarding storage and in-use time periods suitable for using the reconstituted drug product, instructions for reconstitution and dispersion mixing should also be included in the product labeling.

In a clinical setting, several hours might pass between reconstitution/drug preparation and administration. For infusion drugs, the reconstituted liposomal dispersion must be further diluted before administration, and this may take hours as well. The method of reconstitution might necessarily be stated in the product literature or on labels. Commonly used diluents (e.g., 5% dextrose solution, normal saline, bacteriostatic water or sterile water for injection) are described in Section 3.2 above. After rapid and repeatable reconstitution, product should be completely dissolved. For liposomal drugs, rolling vials between palms or hand swirling is better than shaking to avoid excessive stress that leads to dispersion foaming, and resulting liposome physical instability, such as particle size growth [133], and also problems with administration. Reconstituted product foaming, for example, must be dissipated before patient administration.

4.3. Long-term storage of lyophilized liposomal products

Dehydration and desiccation during lyophilization reduces both aqueous hydrolysis and chemical degradation (i.e., lipid peroxidation), and destructive physical alterations to the formulation that compromise the integrity and reliability of the aqueous product. Liposomal product stability is thereby improved. Nonetheless, this chemical and physical stability must be reliable and sufficiently long-term to ensure product shelf-life required for clinical use, regulatory approval and commercialization. Lyophilized liposomal product long term stability depends primarily on each formulation's composition and optimized storage conditions [22]. Therefore, stability testing is a mandatory component of lyophilized liposomal product validation. Accelerated stability studies are commonly used in industry to predict long-term product stability. Accelerated testing at 40 °C at controlled relative humidity of 75% is recommended by the International Conference on Harmonization (ICH) guidelines [134]. Product protections from oxygen and light exposure are routine. Oxygen readily diffuses through glassy lyo-cake sugars [135].

Stability must be tailored to lyophilized product formulation variations. One study examining long-term storage stability of lyophilized liposomal formulations showed that increasing levels of unsaturated lipids increased liposomal lyo-cake degradation, consistent with analogous studies conducted in with aqueous phase liposomal dispersions [136]. Most commercial liposomal products utilize saturated or singly unsaturated lipids for this reason. Lyoprotectant variation affects lyoproduct storage stability as well. Stability comparisons for storing four types of disaccharide- (i.e., trehalose, sucrose, maltose, and lactose) formulated lyo-cakes at various storage temperatures with different RH levels, showed that lyo-cake storage stability depended on disaccharide type [137]. All formulations were destabilized significantly during storage at temperatures above Tg (glass transition temperature). Sugars' capability to depress the crystalline phase transition temperature (Tm) of phospholipid bilayers is well-known [73–81]. The Tg value for the dried amorphous disaccharide-containing lyo-cake product material increases during secondary drying. Product storage below this final Tg is important to ensure lyo-cake stability via maintenance of the cake porosity and rigid-glass cake network structure [138,139]. Temperature excursions above this product Tg allow sugar glasses to undergo a second-order phase transition from a rigid state to a viscoelastic rubbery state, risking collapse of the lyo-cake. Excipients with higher Tg values potentially improve storage stability: the drying solid matrix needs to remain amorphous during freeze drying and storage. Elevated product Tg values that avoid cold chain custody and cold storage are rare but a worthy goal to reduce storage expense and monitoring. Trehalose is commonly recommended as a lyoprotectant because the Tg of pure, dry trehalose is 106 °C, while sucrose has a Tg of 60 °C [140]. Since drying process kinetics are temperature-dependent, lower lyoprotectant Tg or Tc values produce longer lyophilization cycles, increasing costs. Increasing product drying temperature one degree during lyophilization can reduce primary drying time 13% [11]. A temperature difference between the lyo-product Tg and the storage temperature of at least 50 °C has been proposed [11].

Relative humidity (RH) also plays an important role for lyophilized product stability. RH influences product moisture absorption and alters the physicochemical properties of the dry cakes. Lower water content reduces molecular mobility within the dried product [42] and, hence, increases shelf life. Keeping the dried cake dry with low residual moisture is essential. Storage conditions under low RH improve lyo-cake product stability. This includes careful monitoring of final moisture content and maintenance of constant low moisture residuals with quality packaging and storage conditions.

Storage temperature is another key factor for lyo-product storage stability by influencing how residual or entering moisture affects lyo-cake structure. Increasing moisture content reduces product Tg and hence the temperature at which lyo-cake collapse might occur. Kawai et al. tested freeze-dried proteins at three temperatures (20, 40, and 60 °C) at two RH levels (approx. 0 and 32.8%) [137]. At 40 °C, cake collapse occurred at RH = 32.8%, but not at 0% RH. No collapse occurred at 20 °C for RH at either 32.8% or 0%. Product cake destabilization increases with increasing storage temperature and moisture content. As liposomes are increasingly used for delivery of biological drugs (e.g., nucleic acids and proteins), the storage temperature for maximum stability of the encapsulated biologic API must also be considered as well.

5. Application of Quality by Design (QbD) for liposome formulation and process development

Given their empirical nature, liposomal lyophilization studies are highly variable batch processes and therefore often time consuming. As seen in Fig. 3, many different variable combinations must be tested (e.g., ratios between lyoprotectant and drug, between multiple lyoprotectants if more than one is used, different combinations of inner/outer membrane lipids with diverse lyoprotectants, and the freeze-drying processing parameters described in Section 4). Combining these into a formulation matrix then yields many diverse sample formulation properties (e.g., particle size, encapsulation efficiency, encapsulated solute retention (ESR), residual moisture, primary drying time and cake appearance) that require extensive efforts to analyze, compare and validate. Traditional experimental methods are unable to provide a comprehensive understanding of these multi-parametric results. They are unable to identify the complex interactions and effects among tested variables for diverse formulations and processing parameters. Rational elucidation of the product design space is therefore difficult.

Pharmaceutical QbD concepts were first introduced a decade ago by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and United States Food and Drug Administration (FDA) to enable new systematic approaches for developing high-quality pharmaceutical products [141,142]. The QbD strategy seeks to improve scientific understanding of relationships between critical process and product qualities, establish design controls and produce tests based on the scientific limits of understanding variability during the product development phase. QbD methods identify critical product quality and therapeutic characteristics (Quality Target Product Profiles, QTPP), translate them into critical product quality attributes (CQAs) and critical materials attributes (CMAs) desired and then identify critical process parameters (CPPs) to consistently yield the desired drug product exhibiting these desired characteristics. This leads to evolution of the design space, a desired QbD output of the process understanding studies that links defined process input variables and their ranges to ensure consistent quality for large-scale commercial manufacture. Continuous development of this understanding and knowledge space using product data inputs ultimately enables creation of a product design space that initiates with product conceptualization, continues alongside product commercialization, and extends throughout the product life-cycle.

QbD importantly uses real-world knowledge obtained during the product life-cycle to implement continual product improvements. That is, QbD process dynamics evolve and adapt with the product over its life-cycle, using design controls and specifications to first define the product, describe and constrain the variables used to produce the product, validate these specifications with PAT methods, and implement product improvements based on learnings as the product is refined. The QbD process is highly useful for complex products where influences from multi-parametric and variable inputs must be understood to yield consistent, controlled product qualities. QbD workflow is generally summarized in Fig. 4.

Comprehensive QbD approaches generally comprise four key components: (1) a defined product QTPP based on scientifically informed properties and appropriate in vivo relevance; (2) product and manufacturing processes designed to address pre-defined product

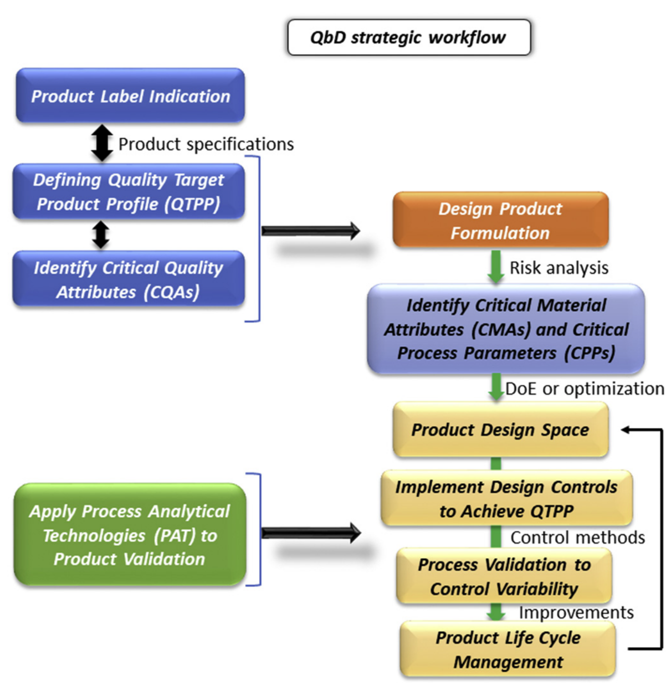

Fig. 4. Strategy for Quality by Design (QbD) pharmaceutical design, control, product validation and improvement process.

performance goals; (3) CQAs, CPPs, CMAs and sources of variability to define the product design space; and (4) manufacturing process controls to yield consistent product quality over time by operating within the established design space. This QbD process assures that pharmaceutical product quality variables are defined, controlled, and verified throughout the process, and via the established design space, provides a multidimensional operational zone of combined variables to produce consistent product quality over time [141,142].

The QbD pharmaceutical development approach to formulation design, product development and manufacturing processes implements quality-improving scientific methods upstream in early research phases so that resulting product quality is designed into product processing at as early a stage as feasible. Design controls are then developed that continually improve the prescribed product quality [141,142]. QbD strategies that address complex drug development variabilities, multiple challenges and quality issues in increasingly complex drug product designs and processes are increasingly used at different stages of pharmaceutical product development. QbD addresses the intrinsic variability associated with 1) batch processing, and 2) multi-parameter, complex mixtures with many formulation meta-stable states and processing conditions. QbD can also exploit Design-of-Experiments (DoE) strategies to address the parameter space and identify critical factors, but QbD is not the same as DoE.

QbD techniques cover all elements of drug development in ICH guideline Q8 [122], now a global expectation from regulatory bodies, also for liposomal products. In this regard, the US FDA finalized its "Guidance for Industry on Liposome Drug Products - Chemistry, Manufacturing, and Controls (CMC); Human Pharmacokinetics and Bioavailability; and Labeling Documentation" in 2018 [132]. The guidance provides recommendations for unique attributes and information that sponsors might best consider for new liposome drug applications (NDAs) and abbreviated new drug applications (ANDAs). The guidance specifically describes these topics for liposome drug products: (A) chemistry, manufacturing, and controls (CMC); (B) human pharmacokinetics and bioavailability or, in the case of an ANDA, bioequivalence, and (C) labeling. Additionally, the guidance mentions descriptions and composition, physicochemical properties, CQAs, description of manufacturing process and process controls, control of lipid components, drug product specification, stability and post-approval changes in manufacturing. Importantly, the FDA guidance does not include recommendations on clinical efficacy and safety studies, nonclinical pharmacology and/or toxicology studies, liposome formulations of vaccine adjuvants or biologics or drug-lipid complexes. Nonetheless, the 2018 FDA guidance refers to QbD principles according to ICH Q8(R2) Pharmaceutical Development, including screening of CQAs and establishing the product design space. Inclusion of detailed process flow diagrams and descriptions of unit operations with ranges for specific process parameters and process controls is recommended, supported by pharmaceutical development data.

In recent years, QbD methods have been increasingly applied to develop freeze-dried pharmaceutical liposomes, especially by industry [38,122]. Since liposome drug products are widely recognized to be sensitive to changes in manufacturing conditions, including batch-batch issues and scaling, process controls are best established during initial product development. Manufacturing process parameters known to affect liposome drug performance are shear force, pressure, pH, temperature, batch size, batch-related hold times, lyophilization parameters, and sterilizing filtration methods. Liposomal QbD approaches must identify characteristics critical for product quality (i.e., QTPP), and translate them into specific attributes required for liposomal drug formulations (CQAs), and then establish how these liposomal formulation and lyophilization process variables are controlled to consistently produce products with the desired profile [141,143]. These elements include QTPP, CQAs, and CPPs, risk assessment methods, developing DOE methods to study the impact of CPPs on CQAs and determine a design space for the liposomal product.

A recent QbD effort for designing freeze-dried liposomes [122] defined drug efficacy and process efficiency as QTPP; and particle size and zeta potential, ESR, residual moisture, Tg, primary drying time and cake appearance all as CQAs. Experiments evaluating formulating impact on these CQAs were conducted by varying lyoprotectants, freezing rate, shelf temperature of primary drying, all with and without an annealing step. By analyzing these results, some variables were confirmed and others excluded, e.g., lyoprotectant type and the applied annealing step. The specific QbD design space was generated by using statistical software after inputting all variables and corresponding responses (i.e., CQAs), providing a threshold to each response, such as setting the ESR to >90%. A design space was shown in a response surface figure generated by using regression models. This region representing the combination of operation variables correlated with a low possibility of failure (<0.5%) can be selected as the general product operation conditions. While several studies report QbD strategies for liposomal formulation development from materials perspectives (i.e., lipid composition, chain length, concentration on resulting particle properties) [9,10,107–111], QbD approaches to liposome lyophilization process complexities are rare. One QbD approach was reported for development of lyophilized liposomes containing simvastatin [38]. Validation of the strategy was further confirmed by preparing a product from a robust setpoint in the design space, with the actual resulting CQAs from this setpoint found within the predicted values from experiments [38]. Importantly, QbD has also been applied to addressing critical variations encountered in scaling lyophilization processes [65]. In summary, QbD is a time-efficient strategy with increasing global regulatory emphasis, holding promising potential to improve and de-risk the complex development processes now required for liposomal lyophilization preparations to yield both controlled and predicable product quality.

6. Regulatory guidance for liposomal drug products

Currently, without liposomal biologic drug products approved and clinically available, liposomal drug products remain a sub-group of Non-Biological Complex Drugs (NBCD), yet with intrinsically greater complexity in characterization and regulatory approvals than traditional small molecule drugs. The first global liposome drug approved for clinical use was the antifungal product, Ambisome™ in 1990 [3]. Due to rapid development of subsequent liposomal products, US FDA and European agencies began to publish guidance discussing recommended or required information that new product applicants should submit for NDAs and ANDAs for liposome drug products during the last decade. The first draft from FDA specific to liposomal drugs was published in 2002; FDA then published a product-specific guidance for PEGylated liposomal doxorubicin (PLD) injectable formulations in 2010 [4]. A much more comprehensive revised draft guidance was issued in October 2015, called "Liposome Drug Products Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation". This guidance under the same name was further updated and finalized in 2018 (as described above) [132]. Given the unique drug-lipid complex mixtures phase states, and behaviors through processing and storage, a liposome drug formulation developer is now expected to devote considerable attention to: 1) product description and composition, 2) physicochemical properties, 3) CQAs, 4) manufacturing processes and process controls, 5) control of lipid components, critical material attributes, CMAs, 6) liposomal product specifications, and 7) formulation stability. To comply, extensive characterization of liposome composition, product variability, and liposomal stability will be required. Details regarding liposome preparation and how non-encapsulated drug is removed and validated are also emphasized required. Liposome-specific difficulties with the sterilization filtration processing must be addressed for parenteral products.

While the 2018 liposome guidance mentions QbD parameters expected for liposomal drug designs, it does not specifically cover lyophilization and lyo-cake reconstitution. It also does not cover liposome drug products seeking approvals under biologics license applications (BLAs). Nonetheless, many guidance principles apply to these complex products. Since QbD mandates and product development pathways and risk analyses compel early decisions for lyophilized formulation when initiating liposomal drug development, regulatory guidance should be clear to facilitate product risk-assessment. Improvements to guidance would facilitate better de-risking of liposomal lyophilized product strategies. Adding product lyophilization later as a post-approval change in manufacturing can lead to new, costly and risky in vivo studies to assess how changes may affect drug performance, stability and shelf-life. Given liposome product complexity, formulation changes made after product approval will require an FDA Prior Approval Supplement.

All current liposomal FDA-approved products are indicated for parenteral use [3], and some as lyophilized liposomal dosage forms, e.g. Ambisome®, Amphotec®, and Visudyne® (see Table 1). Currently, the FDA has issued product-specific guidances for developing generic versions of four of these liposomal products (amphotericin B, daunorubicin citrate, doxorubicin hydrochloride, and verteporphin). Applicants of other generic liposome drug products should use the new final guidance for regulatory advice.

Liposomal products with their heterogeneous compositions, processing and structures do not facilitate complete, convenient physicochemical quality characterization, challenging their regulatory evaluation especially for follow-on product versions upon comparison with the original reference product [144]. In 2017, FDA draft guidance for industry entitled “Drug Products, Including Biological Products, that Contain Nanomaterials” provided needed input on developing human drug products containing a nanomaterial in the final dosage form, addressing a risk-based framework of general principles and specific considerations for quality assessment, nonclinical, and clinical studies as they relate to the development of drug products containing nanomaterials. Specifically, this framework encompasses how manufacturers should characterize the formulation's nanomaterials and possible effects on the drug product CQAs. The FDA does not categorically define products containing nanomaterials as inherently benign or harmful, but rather considers the characteristics of each product when determining product safety for use. Therefore, the FDA requires thorough and comprehensive analyses of new drug products containing nanomaterials. Importantly, this guidance mentions liposomes specifically as nanomaterials, but defers liposomal-specific information to their 2018 FDA guidance. Interestingly, the subsequent 2018 FDA guidance fails to mention nanomaterials in any cross-referencing, confounding possible understanding of the relationships and consistent advisories between these inter-related guidance. Regardless, the FDA's specific focus on the unique safety and efficacy aspects of drugs formulated with nanomaterials is interesting and timely in acknowledging the complexity of these nano-medical products, as well as the challenges associated with approving safe and therapeutically equivalent complex generic versions.

The FDA's continuing ability to establish clear guidelines for developing increasingly complex drugs will be critical for ensuring that regulations keep up with technological advancements and drug product complexity. Specific regulatory guidance and requirements for lyophilized parenteral liposome drug products or emerging liposomal biologics are not available; the assumption is therefore that these regulatory requirements should be the same as for all other lyophilized parenteral products. More extensive and updated efforts from regulatory agencies are needed to match the rapid liposomal product development, and in turn, facilitate and guide the QbD development strategy for future lyophilized liposomal drugs as a complex drug class.

7. Conclusions and expert outlook

After decades of intense liposomal drug development and clinical utilization, the principles, techniques, tools, formulation design and product development pathways required for robust liposomal drug formulations are now much more established. Global compliance with pharmaceutical QbD mandates and ICH guidelines has facilitated standardization and harmonization of design, process validation and formulation assessment. Beginning with the 1990's first successful liposomal formulation clinical entry, parenterally administered liposome-based drugs continue to demonstrate unique properties that overcome some limitations of conventional therapies for select diseases. Much of these therapeutic benefits with small molecule drugs can be attributed to improved drug toxicity profiles and improved maximum tolerated dosing due to altered liposome biodistribution compared to conventional free drugs. However, more recent emphasis on liposomal delivery of biotechnology-based macromolecules (e.g., siRNA, DNA, peptides, proteins) provides evidence for other therapeutic advantages for liposomal delivery.

The parenteral administration pathway is currently preferred clinically for liposomal drugs. Parenteral formulations have specific constraints, unique quality controls and validation protocols. Formulation stability is precarious in aqueous dispersion form. To avoid these known stability issues and the associated logistical problems and costs of cold chain and shelf-life management of liposomal aqueous formulations, lyophilization strategies are common in these formulations. Lyophilization methods exploit previously established knowledge and practices of pharmaceutical lyophilization of traditional non-particle based parenteral formulations (e.g., small molecules, proteins). However, liposome lyophilization has unique technical and formulating considerations to address the lipid vesicle bicompartmental nature of this dispersed aqueous drug delivery system. Vesicle carrier lipid membranes must be carefully handled during freezing, membrane and ice phase transitions, and upon formulation rehydration. They must also reliably reconstitute after drying with unchanged particle properties and high, unchanged fractions of encapsulated drug. Finally, they must exhibit long-term stability as dried cakes under storage conditions, and consistent properties after aqueous reconstitution prior to administration. Collectively, these are constraints not common to traditional drug lyophilized forms, involving many more variables, complex physical and structural interactions over time, space and temperature, and therefore risks of high product variability and instability.

Traditionally, liposomal formulating practices have largely been empirical, guided now by decades of trial-and-error formulating strategies and learnings. Nonetheless, pharmaceutical quality by design (QbD) approaches are well suited to addressing these formulating and quality control complexities and variabilities using more rational processes, quality systems and validated assessment parameters. Liposomal lyophilization methods are seemingly essential to preserving liposome formulation stability and product shelf-life. Improved approaches to understanding the lyophilization variables that impart consistent formulation stability, ESR, CQA and QTPP through known CPPs would provide an enormous boost to liposomal product development. Lyophilization process scaling validation and mechanistic studies for excipient combinations on product reliability should be part of this effort. Given increasing pharmaceutical enthusiasm for pursuing liposomal delivery opportunities and drug delivery technology, regulatory guidance has not kept up with the rapid pace of liposome-based therapeutic developments. Further efforts are needed by regulatory agencies, industry and academia to produce new comprehensive guidance to ensure liposomal product safety, efficacy and drug action for new liposome drug products. Full appreciation of current liposome lyophilization experience now known for these systems and its translation into improved guidance documentation should help advance product development pathways and quality risk management required for new lyophilized liposomal therapeutics required to get new complex products to benefit patients.

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