CHAPTER

19 Size reduction, purification, sterilization and storage/packaging of liposomes

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19.1 Introduction

Doxil was the first liposomal drug delivery system approved by the United States Food and Drug Administration (US FDA) and European Medical Agency (EMA) in 1995 [1]. Doxil is a liposomal formulation of doxorubicin, an anticancer drug that in its free form causes severe cardiac toxicity in humans. Encapsulating doxorubicin in liposomes not only reduces the cardiac toxicity of the drug, but also results in an eightfold increase in the circulation half-life compared to the free drug and increases drug accumulation in tumors [2]. Since its inaugural approval for the treatment of AIDS-related Kaposi's sarcoma, Doxil and follow-up generic versions also have been approved to treat recurrent ovarian cancer, metastatic breast cancer, and multiple myeloma with superior efficacy and safety over other anticancer drug formulations [1].

As of 2022 there were 14 classes of liposomal drug products used in clinics around the world and dozens more in clinical trials [3,4]. There are now decades of research and clinical use that show liposomal drug formulations have many advantages, such as protecting the encapsulated drug products from degradation in the body, extending the circulatory half-life of the drug, and controlling the release of the drug while also showing excellent biocompatibility and safety [3,4]. Liposomal formulations are currently used to improve the pharmacokinetics and pharmacodynamics of drugs for a range of medical treatments including cancer therapies, fungal and viral infections, ocular degeneration, and local pain management [3,5]. Moreover, recent reports suggest that the liposomal drug delivery market will nearly double by 2027, reaching almost $7 billion [3].

Yet liposomal drugs have not advanced to the clinic as quickly as might be expected based on the 50 years of positive results in laboratory studies [6]. Challenges in manufacturing processes and government regulations have been major bottlenecks in the clinical translation of liposomal drug delivery platforms [3,5–7]. Liposomal formulations are chemically and structurally complex. Ensuring the reliability

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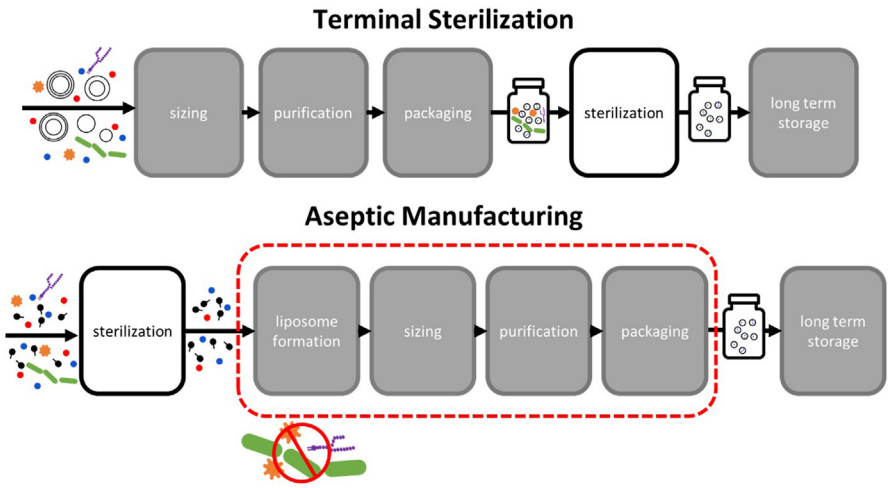

FIG. 19.1

An overview of the liposome manufacturing process including terminal formulation sterilization (*top*) and pre-process component sterilization paired with aseptic manufacturing (*bottom*).

and reproducibility of these complex final products is especially challenging, as industrial-scale production of liposomes requires multiple manufacturing steps and highly specialized pieces of equipment. Many processes used to create liposomes in the laboratory are not readily scalable to industrial processes or are expensive to operate on commercial scales. The manufacturing steps needed to prepare well-defined liposome formulations that meet regulatory requirements can also destabilize or denature the active drug products. The susceptibility of the encapsulated drug products, as well as the components of the liposomes themselves, to degradation also leads long-term instability of the final product and challenges with receiving regulatory approval for clinical use [3,7].

In this chapter, we discuss the four main manufacturing steps needed *in addition* to forming the liposomes, namely (1) size reduction, (2) purification, (3) sterilization, and (4) packaging and storage (Fig. 19.1). *Why* these steps are needed, *when* available methods are amenable to different liposomal formulations, and *how* the steps can be performed on the industrial scale are discussed. Each section is organized in order of industrial relevance in terms of either manufacturing considerations, regulatory compliance, or some combination of the two.

19.2 Liposome sizing

19.2.1 Why?

Liposome sizing refers to the process of reducing the diameter and lamellarity of a pre-existing liposome formulation, also referred to as a liposome suspension.

Typically, unilamellar (i.e., single bilayer) liposomes with diameters of 50−200 nm are desired in drug delivery applications due to their lower clearance, more predictable release kinetics, and more desirable biodistribution compared with larger, multi-layered counterparts [8]. The reduction of liposome diameter during sizing processes is constrained by a critical diameter value (D_c), which reflects the largest curvature (smallest liposome diameter) bilayers can experience without introducing unfavorable molecular packing. The critical diameter of a given liposome system can be theoretically computed using

$$D_c = 2\left(\frac{l_c}{1 - \frac{v}{a_0 l_c}}\right) \tag{19.1}$$

where l_c, v, and a_0 are the critical length, hydrocarbon chain volume, and optimal surface area for a given lipid molecule, respectively, and the nondimensional term $v/(a_0 l_c)$ is referred to as the packing parameter [9]. Note, the majority of liposome systems exhibit D_c values of 10−30 nm.

19.2.2 How?

Techniques for sizing liposomes are generally based upon applying mechanical energy to the initial suspension or forcing it through a confined pathway. The former mechanism relies upon applying enough energy to the suspension such that the bilayer lysis tension—that is, the amount of mechanical energy per unit of surface area that causes the bilayer to fracture ($\approx 10^{-2}$ J/m^2)—is exceeded. As a result, liposomes break apart into smaller bilayer entities referred to as bilayer disks, as well as free lipid molecules [10]. The instability of non-enclosed bilayers (i.e., edge-containing structures) and free lipids subsequently leads to their rapid wrapping and/or reassembly to form smaller, mostly unilamellar assemblies [11,12]. Passage of suspensions through confined pores, on the other hand, is a more complex process and there is conflicting evidence on whether the bilayer bending modulus, which reflects the free energy associated with elastic buckling/bending of the bilayer ($\approx 10^1$ k_BT−10^2 k_BT), plays a role in addition to lysis tension [12].

The industrial relevance of liposome sizing approaches is based upon their scalability and, to a lesser extent, their effect on drug incorporation. In terms of the latter, unit operations based upon rupture and reformation of liposomes (e.g., microfluidization, French press, sonication) enable simultaneous internalization of drug molecules and sizing of liposomes whereas confinement-based approaches (i.e., extrusion) necessitate drug to be incorporated in a separate step before or after sizing. The main processes applied for sizing liposomes currently—in order of decreasing scalability—are microfluidization, extrusion, French pressure cell, and sonication (Fig. 19.2). Details pertaining to each technique will be discussed in the following paragraphs.

Microfluidization is a homogenization technique in which high-pressure pumps and well-designed microchannel geometries are utilized. Microfluidizers are the

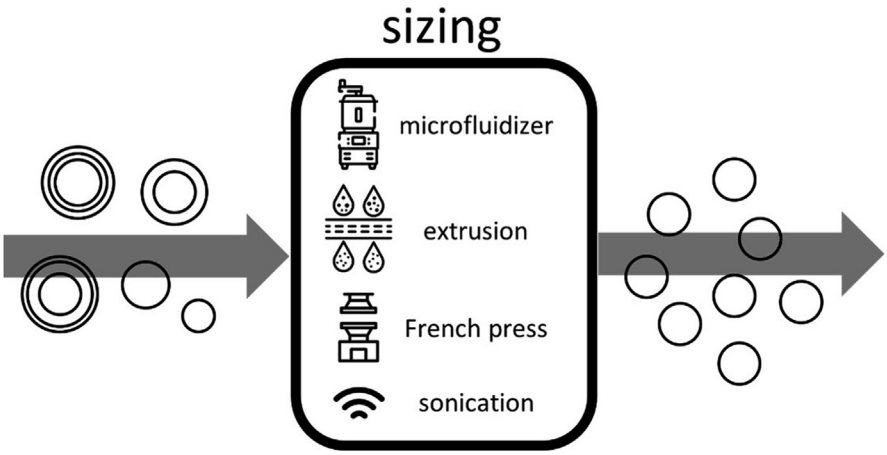

FIG. 19.2

Schematic representation of liposome sizing with relevant process types.

most scalable liposome sizing technique because they can be constructed with increasing numbers of parallel microchannels, which enables consistent processing parameters from laboratory scale ($\approx$1 mL batches) to production runs ($\approx$1000 L/h). Liposome suspensions within the microfluidizer channels experience considerable shear forces and cavitation due to high-pressure operation. Furthermore, contemporary microfluidizer channels are designed such that suspensions are split into two streams, passed through 90-degree junctions, and recombined into a single outlet stream all at high flow velocity resulting in liposome-wall and liposome-liposome impact events [13]. Shear force, cavitation, and impact events all impart the desired mechanical energy for liposome sizing purposes. Unfortunately, there is some concern that these same intense conditions may also lead to chemical degradation of drug compounds, in which case the drug would need to be incorporated after microfluidization-based liposome sizing [14].

The primary microfluidizer operating parameters that can be optimized in liposome sizing processes are pressure, number of recirculation passes, and lipid composition. Early studies [15,16] on the application of microfluidizers found that even one pass at low operating pressure ($\approx$10 MPa) yields a substantial decrease in liposome size ($D \approx 20-60$ nm). Further increasing the number of passes or operating pressure ($\approx$100 MPa) leads to a modest decrease in liposome size and significant reduction in polydispersity index (*PDI*)—which is a measure of the breadth of the size distribution. Alternatively, increasing liposome concentration translates to larger liposome diameters (e.g., an increase from 10 to 100 mg/mL of soy bean lecithin translated to nearly a twofold increase in liposome diameter after one pass) [16], but this effect can be overcome by increasing process intensity (through increased operating pressure or additional passes). Cholesterol-containing liposomes sized via microfluidization exhibit consistently larger diameters than pure lipid analogs.

This phenomenon is not specific to microfluidization and arises due to an increase in average packing parameter with cholesterol addition (see Eq. 19.1). Liposomes containing cholesterol also differ from pure liposomes in that a moderate number of passes seems to be optimal for minimizing diameter (when cholesterol and lipid are present in equimolar amounts). Upon too many passes, cholesterol/lipid mixed liposomes experience regrowth [16]. Poly(ethylene glycol)-coated liposomes (i.e., stealth liposomes) have also been successfully sized down to ≈ 100 nm using microfluidization [17]. In instances of concurrent liposome sizing and drug incorporation, the benefits of increased pressure and number of passes on liposome diameter must be balanced with drug encapsulation efficiency, which has been shown to decrease with increases of these operating parameters [16].

Unlike microfluidization and the unit operations discussed below, liposome extrusion does not depend solely on mechanical energy as a means to reduce liposome diameter but also relies upon confinement of the lipid assemblies within nanoscopic ($\approx 10-100$ nm) pores. Typically, the liposome extrusion process proceeds by passing a liposome suspension through a polycarbonate membrane, or series of polycarbonate membranes, with well-defined cylindrical pores (usually produced via track etching) [18]. Lab-scale liposome extrusion devices ($\approx 0.1-1.0$ mL) are constructed of a sealed, dual-syringe setup wherein a suspension is expelled from one syringe through the polycarbonate membrane and into the other syringe. Subsequent passes can then be carried out by simply expelling the suspension from the second syringe through the membrane back to the first syringe. The force applied to pass fluid through the membrane can either be manual (i.e., by hand) or automated (e.g., using a syringe pump). Larger-scale liposome extruders ($\approx 10-1000$ mL) are composed of a sealable, stainless steel cylinder with a polycarbonate membrane in the base. The liposome suspension is placed within the cylindrical chamber and is forced through the membrane via compressed air or nitrogen. For subsequent passes, the suspension exiting the extruder must be reloaded into the original chamber, or multiple extruders can be set up in series.

The primary operating parameters for liposome extrusion are membrane pore size, extrusion pressure, and number of passes. The number of passes and extrusion pressure each lead to smaller liposomes [19]. Moreover, membrane pore size directly dictates liposome size, particularly following several passes [19,20]. Due to liposome size dependence on pore size and the fact that pore size typically exceeds their critical diameter by a comfortable margin, suspension composition has little effect on liposome diameter following the extrusion process. Composition does however affect the temperature and pressure required to pass suspensions through the polycarbonate membrane (at a constant volumetric flow rate). For example, suspensions with higher liposome concentrations (up to ≈ 50 mg/mL) and liposomes containing cholesterol tend to require greater pressure for extrusion due to increased bilayer concentration and lysis tension/bending modulus of the bilayer, respectively [21].

The French pressure cell (i.e., French press) is a device that was originally designed to isolate and study proteins and other cellular components by stripping

away the cell plasma membrane. The French press, in the simplest sense, is a cylindrical chamber with a piston and external hydraulic pump. A liquid suspension is placed within the cylindrical chamber and pressurized with the pump-driven piston. A small orifice or needle valve regulates flow of the suspension from the bottom of the press and, as a consequence, the pressure within the cylinder. At the outlet of the French press, the suspension experiences rapid decompression—causing cavitation—and high shear, which are the sources of energy that lyse lipid bilayers whether they are cell membranes or liposomes. The French press method is restricted to $\approx 1-10$ mL batch scale and so has been used exclusively in laboratory settings.

The operating parameter dependence of the French press is comparable to microfluidization with process parameters including pressure, number of passes, and suspension composition. A single pass at high pressure (≈ 140 MPa) yields a broad distribution of liposome sizes that can be reduced to a suspension of small, unilamellar liposomes ($D \approx 20$ nm) following centrifugation. Subsequent passes (2–4) leads to a reduced liposome *PDI*—such that no centrifugation is needed for size refinement—with a similar liposome diameter range. This method is also similar in that increased process intensity (i.e., operating pressure and number of passes) enables even high liposome concentrations (≈ 100 mg/mL) [22] to be sized effectively. However, the French press method for liposome sizing is more sensitive to pressure than microfluidization. Experimental observations suggest that even reducing pressure down to ≈ 100 MPa greatly decreases the formation of small, unilamellar liposomes [22]. Similarly, the inherent change in bilayer properties with added cholesterol causes larger liposomes ($D \approx 30$ nm) to be formed via the French press method even when only small amounts of cholesterol are added [23].

Sonication, which consists of exposing suspensions to ultrasonic waves, applies mechanical energy solely in the form of cavitation as a means to reduce the size of liposomes. Cavitation during sonication occurs due to movement of the ultrasonic pressure waves through the suspension, which can be conceptualized as cyclic compression and expansion at a given location. These pressure swings cause bubble formation, growth, and collapse within suspensions, and the intense collapse of bubbles is the source of energy transfer toward liposome lysis. Ultrasonic waves can be delivered to liposome suspensions via probe or bath sonication. Probe sonication delivers ultrasonic waves directly from an ultrasonic generator to the suspension, and as a result, is a high intensity, localized form of sonication. It has been shown that the direct contact between probe and suspension in probe sonication can lead to metallic contamination of samples, which is undesirable particularly in pharmaceutical formulations. Additionally, the intense energy transfer causes chemical degradation of lipids in some cases (particularly unsaturated lipids) [24]. Bath sonication is an indirect means of ultrasonic wave delivery consisting of an ultrasonic generator that transfers waves to a water bath. The ultrasonic waves can be subsequently transferred to suspensions in sealed vessels by submersion in the water bath. Bath sonication delivers lower intensity ultrasonic waves over a larger affected volume but with inconsistent distribution and repeatability. Scale-up of sonication processes is

complex due to altered geometric factors when sample volume/throughput is increased, and commercial scale processes are mostly probe-based. As a result, there is little evidence of production-scale sonication processes for liposome sizing. The bath-based form of the technique has been used to prepare lab-scale batches of ≈1−10 mL and has been proposed as a point-of-use preparation scheme [25,26].

The primary sonication operating parameters for liposome sizing are ultrasonic wave intensity and frequency, sonication time, and suspension composition [27]. Similar to the previously discussed techniques, increasing process intensity (ultrasonic wave intensity) and process longevity (sonication time) generally lead to a decrease in liposome size and each can be further increased to reduce liposome size at high liposome concentrations [27,28]. The relationship between ultrasonic frequency and resultant liposome diameter is less straightforward. Basic wave principles would suggest that higher frequency waves should yield smaller liposomes since they inherently carry more energy. It turns out that the opposite is observable: decreasing frequency by an order of magnitude (≈400 to ≈40 kHz) translates to considerably smaller liposomes [27]. Instead of wave energy, the explanation lies in that lower frequencies—and hence longer wavelength pressure waves—enable larger bubbles to form within the suspension and, upon cavitation, these larger bubbles create higher local energy. Higher frequency is, however, more likely to degrade lipid molecules and subsequently disrupt the liposome structure—a likely outcome of the increased wave energy. Concurrent drug incorporation and liposome sizing can also be achieved at a moderate level using bath sonication [26], but the chemical stability of all components must be carefully considered.

In addition to the aforementioned sizing practices, there are a few liposome fabrication techniques that produce small, unilamellar liposomes directly and, therefore, do not require a subsequent sizing step. Notably, a process referred to as micro-hydrodynamic focusing consists of flowing a lipid-alcohol suspension between aqueous layers within a microchannel. Solvent exchange at the liquid-liquid interface leads to bilayer formation and successive liposome production. Liposomes leaving the micro-hydrodynamic focusing process are unilamellar and can have diameters in the range of 40−140 nm with control over the specific output diameter based on the ratio of aqueous flow to lipid-alcohol solution flow [11]. More on the topic of microfluidic methods for liposome formation can be found Chapter 21. There has also been recent exploration on the direct production of small, unilamellar liposome using supercritical CO_2, but this avenue has yet to yield small liposomes of desirable PDI. Additional information on the use of supercritical fluids in liposome formation can be found in Chapter 22.

19.3 Liposome purification

19.3.1 Why?

Liposome purification refers to the separation of drug-loaded liposomes from unentrapped drug and any organic solvent or surfactant used in upstream preparation.

Purification is crucial in the preparation of liposome-based products because of the implications of composition and impurity presence on product efficacy and associated health risks. This point is highlighted by regulatory controls on related factors such as overall composition, quantities of encapsulated and free drug, and presence of residual non-aqueous solvent in liposome formulations (see for example the U.S. Food and Drug Administration's Guidance Document on Liposome Drug Products) [7].

19.3.2 How?

Separation of formulations into fractions containing their desired and undesired species requires physicochemical differences between those species. Usually, liposomes are considerably larger than drug compounds and surfactants that may be present in formulations, which leads to the use of several size-based separation operations including size-exclusion chromatography, ultrafiltration, and dialysis. It is also common for the molecules comprising liposomes and drugs to possess opposite charges. In these cases, charge-based separations like ion exchange chromatography can be effective. Finally, density differences between liposomes (higher) and unencapsulated drug (lower) can be leveraged for separation using centrifugation.

As with liposome sizing, the industrial relevance of liposome purification processes is primarily based upon their scalability. Additionally, certain purification processes (like, ion exchange chromatography) are only relevant in specific instances (e.g., when a charge difference between liposomes and unencapsulated drug exists) and so must be considered carefully whereas methods based on size contrast are broadly applicable. The methods of purifying liposomes—in order of decreasing scalability—are size-exclusion chromatography, ion exchange chromatography, ultrafiltration, dialysis, and centrifugation (Fig. 19.3).

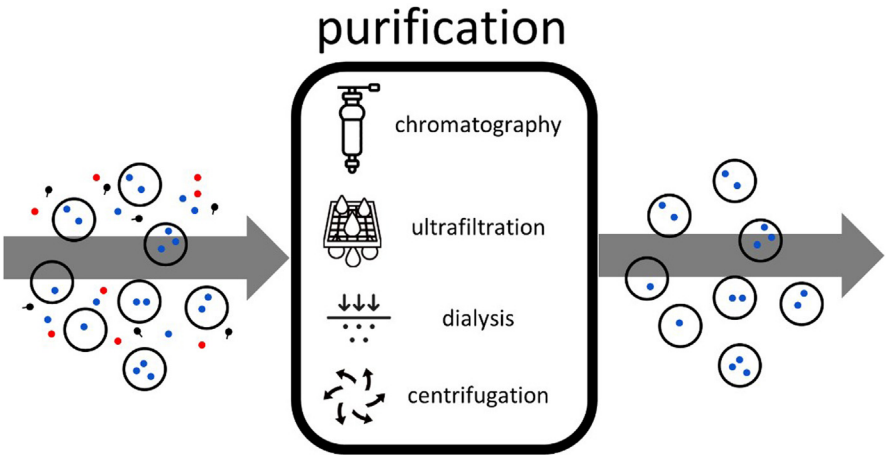

FIG. 19.3

Schematic representation of liposome purification with relevant process types.

Size exclusion chromatography (SEC), also referred to as gel filtration chromatography when used for fractionating aqueous systems, operates under the principle that aggregates permeate through a gel-packed column at different rates based upon their size. Specifically, small aggregates traversing the chromatography column have access to the majority of pores within the gel media and so follow a more tortuous path. Larger aggregates, on the other hand, cannot enter smaller channels due to steric exclusion and therefore elute through a more direct route. These phenomena lead to larger aggregates passing through the SEC column quicker while small molecules and aggregates are retained longer. As a result, fractions of liposome formulations eluted at short retention times are reserved as purified liposomes. The smaller, undesirable components, which are eluted later, are discarded or recycled as appropriate.

A considerable advantage in using SEC for liposome purification is that the formulation media does not need to be changed for the purification process. The desired pH, salinity, etc. can be retained without impacting the phenomena that drive purification nor adversely affecting the materials comprising the SEC equipment. Furthermore, SEC is mild in terms of the forces (mechanical, osmotic, etc.) that it exerts on liposome formulations since it does not require external pressure or special formulation conditions (e.g., salinity). The first consideration for purifying a specific liposome formulation using SEC is selection of an appropriate gel bead media. Several classes of gel bead media are available including Sephadex (a dextran-based gel), Sepharose, Bio-Gel A (agarose-based gels), and Sephacryl (a dextran-polyacrylamide gel) [29].

Aside from their unique chemistries, the various classes of gel bead media have different pore sizes and pore size distributions. Pore size and pore size distribution are the primary factors used to control the size range over which separation occurs and the size resolution of that separation, respectively. Sephadex gel beads generally have smaller pores ($\approx 10^3$ g/mol-10^6 g/mol) and narrower pore size distributions ($\approx 10^4$ g/mol-10^6 g/mol) whereas Sepharose and Bio-Gel A typically have larger pores ($\approx 10^5$ g/mol-10^7 g/mol) and broader pore size distributions ($\approx 10^6$ g/mol-10^7 g/mol). Note, industry standard is to present pore size information as the molecular weight range segregated by pores. Bead diameter (i.e., fine [$\approx$20–80 μm], medium [$\approx$50–150 μm], or coarse [$\approx$100–300 μm] gel particles) can also have some effect on the resolution of SEC purification. Liposomes are large relative to even the biggest gel pore sizes and so are mostly to fully excluded from pores. For example, liposomes with diameters of >60 nm and >100 nm are fully excluded from Sepharose 4B (6×10^5 g/mol-2×10^7 g/mol) and Sepharose 2B (7×10^5 g/mol-4×10^7 g/mol) gel beads, respectively [30]. Therefore, gel selection is commonly focused on including undesired components' in pores, avoiding liposome retention, and maximizing formulation flow rate through the column. Larger pore sizes, larger bead diameters, and lower gel bead volumes (within the column) are employed to address these factors [31]. In specific applications requiring narrow liposome size distributions, SEC can also be applied to purify size-specific, loaded liposomes using very large pore size gel media (e.g., Sephacryl S-1000) [32].

Like SEC, ion exchange chromatography (IEC) is used to purify formulations by passing them through a column containing gel beads. However, the purpose of the gel beads in IEC is to bind with ionic compounds of specific charge (i.e., positive or negative) while neutral compounds or those with the opposite charge (i.e., negative or positive) pass through the column [33]. Gel beads for IEC are composed of insoluble, charged polymer resins. In this case, the bead pore size and diameter are simply used to control the surface area of the resin since the ion-exchange process mostly occurs at the formulation-resin interface. In the context of liposome purification, cationic IEC is the preferred method of ion exchange since the majority of liposomes are neutral or negatively-charged whereas many drug molecules (e.g., doxorubicin) and some surfactants (e.g., tetradecyltrimethylammonium bromide) are positively-charged [34]. Cationic IEC uses acidic polymer resins that bind positive counter-ions. Upon exposure to a liposome formulation including positively-charged, unencapsulated drug, the ion-exchange resin releases its initial positive counter-ions in exchange for those drug molecules. As a result, loaded liposomes are eluted from the column quickly whereas unloaded drug is retained or released much slower. One limitation in using IEC for liposome purification is that, depending on the ion-exchange resin, the formulation content can impact the efficacy of the binding process. For example, formulations with high salinity shield the ionic interactions that cause drug to bind with the ion-exchange resin and decrease the overall ion exchange process, which may necessitate alteration of formulation composition prior to IEC.

Ultrafiltration and dialysis are similar separation techniques in that they both rely upon semipermeable membranes for purification of liposomes. In each case, smaller compounds and ions are able to transverse the semi-permeable membrane (pore size $\approx 1-10$ nm) while the larger liposomes cannot. The liposome-rich stream exits the membrane unit as retentate—the stream not passed through the membrane—and the undesired components leave as permeate—the stream having passed through the membrane. The primary operational difference between the two techniques is that ultrafiltration utilizes a transmembrane pressure gradient—and is therefore similar in operation to liposome extrusion—to drive the separation process whereas dialysis takes advantage of a transmembrane concentration gradient [35]. The pressure gradient in ultrafiltration leads to simultaneous purification and concentration of liposomes due to passage of smaller compounds and water through the membrane, and the post-ultrafiltration retentate can be diluted to the desired liposome concentration if necessary. Alternatively, a nearly equal exchange of water molecules occurs through the membrane during dialysis and so liposomes are only purified. Each process can be carried out in the desired aqueous medium with minimal consequence (as long as the process is well-designed). However, depending on the chemistry and composition of the liposome formulation, the magnitude of transmembrane pressure required for ultrafiltration ($\approx 0.1-1.0$ MPa) can negatively affect the separation process by essentially extruding liposomes through the membrane. Dialysis is free from this concern since it does not rely upon a pressure gradient, but it typically requires more time for sufficient purification.

Membranes for ultrafiltration and dialysis are composed of polymers such as cellulose acetate, regenerated cellulose, polysulfone, polycarbonate, or poly(vinylidene fluoride). Ultrafiltration can also be performed with membranes composed of ceramics like aluminum, zirconium, and titanium oxides [36]. Membrane material selection is primarily dependent on the composition of the solution. In most cases, cellulose-based materials are selected for purifying aqueous solutions due to their high hydrophilicity, low cost, and sustainable sourcing. Ceramic membranes are also regularly used (for ultrafiltration) due to their longevity compared with polymeric analogs.

The mechanisms of ultrafiltration include direct-flow, tangential-flow, and batch filtration. Direct-flow and tangential-flow filtration are continuous processes, which are more amenable to large-scale operations. The former is carried out with the formulation inlet/retentate and permeate flowing in the same direction parallel to the membrane but on opposite sides. The latter consists of the formulation inlet/retentate flowing parallel to the membrane while permeate is withdrawn perpendicularly. Finally, batch filtration consists of loading a formulation into an enclosed chamber partitioned by a membrane. The upstream side of the chamber is pressurized; then, purified liposome retentate and permeate are extracted from the upstream and downstream side of the chamber, respectively.

The dialysis process can also be conducted in continuous or batch operation. Continuous dialysis processes are of more value commercially, again due to scalability, and commonly employ hollow fiber modules wherein a liposome formulation flows through hollow fiber membranes and small compounds and ions permeate into the dialyzing fluid in the module's main chamber [37]. In contrast, batch dialysis is conducted by loading a liposome formulation into a dialysis membrane sack and submersing the sack in dialyzing fluid. Small molecules and ions permeate into the dialyzing fluid, which is regularly exchanged to maintain a concentration gradient, and the purified liposome formulation is extracted from the membrane sack after a sufficient number of fluid exchange cycles.

Centrifugation fractionates formulations by spinning them around an axis at high speed to expose the dispersed components to a considerable centrifugal force. Large, high-density species migrate toward the periphery (i.e., bottom) of the centrifuge container more rapidly than smaller, less-dense species as a result. The intensity of this process is described by the centrifugal acceleration applied to the formulation relative to gravity, g, and the optimal centrifugal acceleration depends on the density ratio between the compounds to be separated, as well as, the density of the formulation medium. Lower density contrast and higher density media require greater centrifuge speeds ($\approx 10^5$ g) while high density contrast formulations in low density media can be successfully separated at as low as $\approx 10^2$ g. Liposome formulations are usually centrifuged at the upper end of this spectrum ($\approx 10^4$ g) to achieve separation from unentrapped compounds, and the resultant "pellet" product is highly concentrated in liposomes. The "pelletized" liposomes can be subsequently diluted to the desired concentration after centrifugal purification. Unfortunately, centrifugation can lead to liposome instability from the high applied force and is difficult to run in a continuous mode when purifying liposomes, which limits scalability.

As a final note on liposome purification, the techniques discussed above can be carried out simultaneously or in series. For example, centrifugal SEC purifies formulations using the centrifugation technique in concert with specialized centrifuge containers containing gel beads through which size exclusion separation occurs [38]. Alternatively, unit operations in series can include several of the same purification technique or a combination of different ones to achieve higher purity liposomes, less intense processing conditions, and/or greater economic feasibility. As an example, efficient production of high-purity liposomes may occur through tangential-flow ultrafiltration (to rapidly remove small impurities) followed by SEC (to remove larger unentrapped drugs and dilute the formulation to its desired concentration).

19.4 Sterilization

19.4.1 Why?

In the pharmaceutical industry, sterilization refers to a process that removes, kills, or deactivates all microorganisms and biological agents in a formulation. Regulations require that all parenteral drugs—where a parenteral drug is one delivered via injection—are sterilized to prevent disease transmission due to usage of the drug. Almost all liposomal drug formulations currently on the market are intended for parenteral delivery, making sterilization an essential step in liposome production.

Sterilization is typically quantified by what is known as a Sterility Assurance Level (SAL). Because it is impossible to prove that all microorganisms have been killed or all biological agents are deactivated, the SAL is a statement of probability that there is a single microorganism remaining on an item after the sterilization treatment [39]. An SAL $\leq 10^{-6}$ is typically required for a product to be labeled as "sterile", where a value of 10^{-6} corresponds to a theoretical probability of one in a million that a viable microorganism is present in the product [40,41]. Note that SAL is also sometimes used to describe a reduction in the microbial content as a result of a sterilization process (e.g., a process that results in a 6-log reduction in microbial content might be said to have an SAL of 10^6). To avoid potential confusion regarding the definition of a SAL, some publications instead discuss the probability of nonsterility or the probability of a nonsterile unit [42].

The most important industrial consideration in selecting a sterilization process is regulatory compliance. Major regulatory agencies have preferred approved methods, and the EMA even publishes well-defined decision trees for selecting the appropriate sterilization process [40]. Approved sterilization methods—in order of decreasing preference by regulatory agencies—include steam sterilization, dry heat sterilization, ionizing radiation, chemical sterilization, and sterile filtration (Fig. 19.4), as well as aseptic manufacturing processes (Fig. 19.1). However, many conventional sterilization methods have been shown to result in chemical and/or physical degradation of liposomes as recently reviewed by Toh and Chiu [43] and Luc Delma et al. [44], and sterilization of liposomal formulations can be a major challenge.

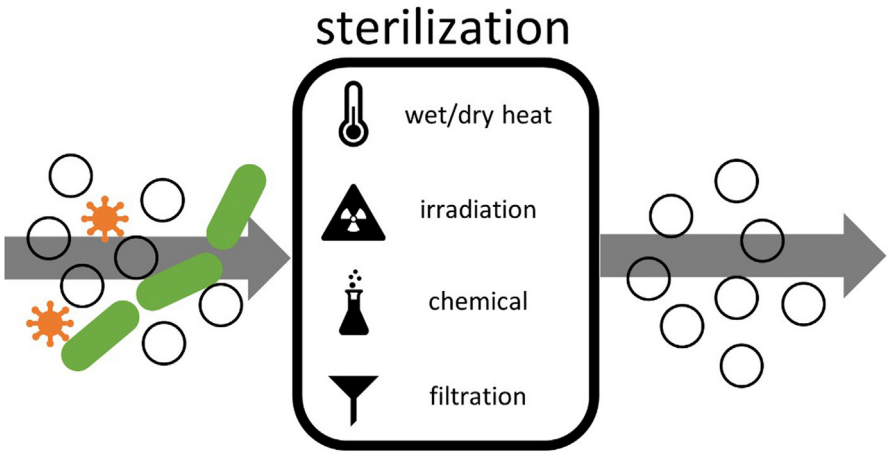

FIG. 19.4

Schematic representation of sterilization with relevant types of terminal sterilization.

Given the susceptibility of liposomes to degradation, selection of a sterilization process requires two major considerations, (1) the process must meet the minimum SAL requirements and (2) the process does not affect the liposome integrity or the activity of the final formulation.

19.4.2 When?

The regulatory decision trees for selecting the sterilization method also determine *when* the sterilization process is performed in the manufacturing process. The decision trees start with *terminal sterilization,* a process in which the formulation is sterilized in its final container as the last step of the manufacturing process (Fig. 19.1). While terminal sterilization is the preferred sterilization method of regulatory agencies, it is the least suitable option for liposome formulations.

If terminal sterilization is not an option, the next preferred method is *sterile filtration*, where the drug-loaded liposome formulation is passed through a sterile filter and loaded into a pre-sterilized container using an aseptic filling process. Most commercial liposome formulations are prepared using sterile filtration, though there are still several considerations with this method. When a formulation cannot be sterilized by any other method, *aseptic manufacturing* is used as a last option to ensure drug products are sterile. In aseptic manufacturing, the raw materials are sterilized and then processed in a sterile environment as shown in Fig. 19.1. An important distinction is that aseptic manufacturing is not a sterilization process, but the usage of sterile equipment and processes to avoid microbiological contamination. Combining an aseptic manufacturing process with a terminal sterilization step is recommended by some as a best practice for patient safety because a less aggressive terminal sterilization method can be used after aseptic manufacturing, and therefore,

the terminal sterilization process may have less adverse effects on the final liposomal drug product [42].

19.4.3 How?

Steam sterilization (i.e., autoclaving) is the first method of choice in the EMA guidelines for the sterilization of medicinal products, active substances, excipients, and primary containers because it is a safe, cost-effective and well-characterized process [40]. Steam sterilization involves treating drug products under pressurized, saturated steam conditions at high temperatures. These conditions are known to lead to hydrolysis of proteins, and therefore, are very effective in deactivating viruses and microorganisms. Unfortunately, these conditions also result in hydrolysis of lipids. Hydrolysis of lipids has not only been shown to lead to physical degradation or aggregation of the liposomes, but also leakage of the drug product [45]. Therefore, aqueous liposomal formulations are usually not amenable to steam sterilization; however, steam sterilization can potentially be used to sterilize the raw materials used in aseptic manufacturing processes or the final containers used in aseptic filling processes.

Dry heat sterilization is also commonly used as a terminal sterilization process for dry powder or semi-solid drug products but is not suitable for most liposomal products. Dry heat sterilization involves extended exposures to high temperature ($>160^{\circ}C$) for prolonged periods of time (>2 hours) [40]. These conditions would result in evaporation of aqueous liposomal formulations but could potentially be used as a terminal sterilization method for lyophilized liposomal cakes depending on the lipid composition. Dry heat sterilization processes primarily work by inducing oxidation, which is also one of the main degradation mechanisms of unsaturated lipids. Accordingly, dry heat sterilization should be avoided for liposomal formulations containing unsaturated lipids.

When heat treatment is not an option, ionizing radiation is another commonly employed method for the sterilization of dry powder or semi-solid drug products. The most widely used method is gamma (γ) irradiation methods that use high energy gamma rays produced by the Cobalt-60 or Cesium-137. The high energy gamma rays remove electrons from water to generate free radicals, and the generated free radicals then alter nucleic acids, which in turn leads to death of microbial contaminants. However, these free radicals can also catalyze the degradation of lipids. Several studies have investigated the potential use of antioxidants to minimize the effects of the generated free radical on the liposomes [26], but such additives do not compensate for the extensive lipid peroxidation in aqueous formulations, especially in liposomes prepared with unsaturated lipids. There are some studies that suggested γ-irradiation could be used to sterilize liposomes prepared with saturated phospholipids [28] or lyophilized liposomal formulations [26,46], as removing the water from the formulation removes the major source of free radicals. The efficiency, high-penetrating power, and well-documented effectiveness also make γ-irradiation a useful sterilization method for heat-sensitive raw materials in aseptic manufacturing processes.

Like γ-irradiation, chemical sterilization can be performed at low temperatures for heat-sensitive drug products. One of the most widely used chemical sterilization agents in the pharmaceutical industry is ethylene oxide. Ethylene oxide is an alkalizing agent and is effective against a wide spectrum of bacterial as well as viral contaminants. Because of this broad effectiveness, ethylene oxide is used to sterilize almost half of the approved medical devices in the United States [47]. However, ethylene oxide is also a known carcinogen and mutagen, and the EMA only accepts ethylene oxide sterilization when no other method for terminal sterilization is possible [40]. It is also important to note that ethylene oxide could only be used to sterilize lyophilized liposomal formulations, as ethylene oxide rapidly degrades in aqueous solutions. Literature studies further support that ethylene oxide treatment may be a suitable terminal sterilization method for lyophilized liposomal formulations [48].

With growing concerns about the environmental impact of ethylene oxide, the US FDA recently announced a public innovation challenge to identify new sterilization methods and technologies to replace ethylene oxide [49]. Several of the technologies selected in the US FDA challenges are chemical sterilization methods including nitrogen dioxide, vapourized hydrogen peroxide, vapourized hydrogen peroxide-ozone, and supercritical CO_2 sterilization methods. While there is some published information validating the efficacy of these non-traditional chemical agents, there is not a long record of safe and effective use, and many of these emerging chemical sterilization methods have not been explored for use on drug products, let alone liposomes. Among new sterilization methods, supercritical CO_2 has emerged as a promising sterilization agent for temperature- and chemically-sensitive products, such as liposomes [26,27]. Recent studies showed that supercritical CO_2 could be used to treat liposomal formulations and achieve an SAL of 10^{-6} without changing the physical appearance of the formulation or causing significant degradation depending on the lipid composition. As with many of the sterilization methods, saturated phospholipids were not affected by the supercritical CO_2 treatment, while unsaturated lipids were significantly oxidized [50].

When terminal sterilization using dry/wet heat treatments, ionizing radiation, or chemical treatment are not possible, sterile filtration of the aqueous liposomal formulation is the next best option. Sterile filtration involves passing the formulation through a sterile filter with 0.22 μm diameter pores to physically remove biological contaminants—much like ultrafiltration processes used to purify liposome formulations but with liposomes passing through the filter as permeate and biological contaminants being rejected as retentate. Accordingly, sterile filtration can only be used for formulations where the liposomes' diameters are $<$200 nm. Some studies have also shown that the filter material can affect the stability of the final product [51]. Other limitations are that sterile filtration does not remove bacteria or viruses that are smaller than the filter pore size, can result in large product losses, and requires high-pressure operation that can be expensive [26]. Moreover, components of liposomal formulations can interact with and clog the filter matrix, and as such, the Guidance for Industry published by US FDA on Liposome Drug Products

recommends that manufacturers demonstrate the microbial filters used to sterilize the products function correctly and do not compromise the integrity and structure of the liposomes [7]. Despite these challenges, sterile filtration is still the most prevalent sterilization method used in academic and industrial formulation of liposomal drug products.

When no other sterilization methods are viable, aseptic manufacturing is the method of last resort because these methods are especially challenging to implement and verify. Aseptic manufacturing is defined as the use of "technological controls to process sterile components while avoiding the introduction of additional microbiological contaminants." [40] In aseptic manufacturing, all raw materials are sterilized using one of the processes described above, and then the liposomes are formed, processed to the desired size, purified, and packaged in a sterile container in isolated environments with minimal interaction from personnel through the use of isolators or restricted access barrier systems. All process equipment is sterilized prior to manufacturing runs, and current good manufacturing practices for aseptic processes require that $\geq$99% of all microbial samples taken in manned aseptic environments (e.g., from air, surfaces, and personal) must be free of contamination [42]. As such, aseptic manufacturing processes are expensive to implement and subject to strict regulatory requirements [52]. For example, because the SAL of an aseptic manufacturing process cannot be directly measured, aseptic manufacturing process validation requires running the process with growth media instead of drug product, which takes significant amounts of times and reduces the overall output of the final product [53].

While sterilization of liposomal drug products using traditional methods remains a major challenge, advances in continuous manufacturing processes and automation technology are especially promising for complex formulations such as liposomes. Personnel are the most significant contributors to microbial contamination, and as such, advances in automation and robotics are enabling the development of so-called "advanced" aseptic manufacturing processes that do not require human intervention and thereby minimize the risk of microbial contamination. Likewise, the development of continuous manufacturing processes that limit the number of process start-up and shutdown operations that are often the source of microbial contamination simplify the design and operation of aseptic manufacturing processes, but also makes the identification of unique product "batch" numbers a challenge which can complicate sterility assurance and conforming to regulatory requirements [53,54].

19.5 Packaging and storage

19.5.1 Why?

Once the liposomal drug product is manufactured, it must be packaged and stored. The packaging and storage conditions are chosen to maximize the *shelf-life* of the drug product, which the Internal Conference on Harmonisation of Technical

Requirements for Registration of Pharmaceuticals for Human Use defines as the "The time period during which a drug product is expected to remain within the approved shelf life specification, provided that it is stored under the conditions defined on the container label" [55]. Long-term stability studies must be submitted to regulatory agencies as part of the approval process to define the shelf-life specifications based on critical quality attributes of the liposomal formulation, which may include specifications of the liposome size and surface charge, quantities of lipid degradation products in the formulation, or drug retention over extended periods of time [55,56].

Product shelf-life is determined as the time point at which the measured critical quality attribute exceeds the acceptance criteria. For example, upper and lower acceptance criteria might be defined as values of 105% and 95% of the value on the label, respectively [57,58]. The time point at which these criteria are exceeded is defined as shelf life of the product and is listed as the expiration date of the drug product label [57]. Regulatory agencies around the world require that the expiration date is directly indicated on the drug container label [58], and given the susceptibility of liposomes to degradation, selecting appropriate packaging and storage conditions is challenging yet essential to ensure the efficacy and safety of the final product.

All lipids will chemically degrade given sufficient time. The rate of the chemical degradation depends on the lipid composition, composition of the formulation medium, and additives in the formulation as well as the storage conditions such as the temperature, exposure to light, and presence or removal of air and/or humidity. Hydrolysis of the ester linkages connecting the lipid headgroup to the fatty acyl tails is accelerated in the presences of acids or bases and leads to the formation of free fatty acids and lysolipids [59]. On their own, lysolipids have been shown to be cytotoxic and have emerging roles in health and disease [60,61]. Oxidation of the double bonds in unsaturated lipids is accelerated in polyunsaturated lipids and by reactive oxygen species—such as hydroxyl radicals—as well as in the presence of UV light and oxygen [59]. In the body, oxidized lipids that form in injured tissues are important regulators of the inflammation and immune response, which has recently led to the hypothesis that the presence of oxidized lipids is an indicator of a threat to the host [62]. Given the potential adverse effects of the degradation products, the US FDA recognizes free-fatty acids, peroxides, and lysophospholipids as impurities [56], and the quantities of these degradation products in the liposomal formulation must be measured, tracked over time, and reported as part of the stability studies required for final regulatory approval. Hydrolysis and oxidation of lipids also has been shown to lead to changes in the liposome size, shape, polydispersity, and surface charge as well as lead to leakage of drug products that can all affect the efficacy and safety of the drug product [63].

When selecting packaging and storage conditions for liposomal drug products, manufacturers must consider the long-term stability of the drug product as required by regulatory agencies, ease of manufacturing, as well as transport and supply chain considerations. Given these considerations, current storage conditions—in order of

increasing liposomal chemical stability but at the expense of increasing formulation and manufacturing complexity—include storage of formulations at refrigerated temperatures, storage of frozen formulations at temperature at or below −20°C, and storage of lyophilized liposomal cakes.

19.5.2 How?

Most of the liposomal formulations currently on the market are sterilized by filtration and then packaged into pre-sterilized primary containers such as vials, bottles, or bags, using an aseptic filling process. After filling, the primary containers are sealed using sterilized stoppers, aluminum seals and closures, screw caps, or heat-crimping methods. In some cases, the primary container containing the liposomal formulation is also sealed in a secondary container [64]. The sealed products are then transferred to the appropriate storage conditions.

Whenever possible, liposome formulations are prepared to be stable at 4°C. The majority of approved liposomal products on the market are packaged as liquid formulations because this minimizes the number of manufacturing steps and simplifies end-use of the product [4]. Storing products at 4°C reduces the cold storage chain requirements which reduces costs and helps ensure more equitable drug product distribution. Unfortunately, these conditions are also most likely to lead to rapid liposome degradation and are not suitable for many formulations. The long-term stability of liposomes in suspension at cold temperatures depends on the lipid composition, formulation conditions, as well as additives used to stabilize the suspension [59].

The primary parameters that can be optimized for storage at 4°C are the lipid and media composition. The use of saturated lipids reduces the risk of oxidation and slows chemical degradation, though some studies have shown that even saturated phospholipids can be oxidized at high temperatures [59]. Likewise, formulating liposomes with saturated lipids that often have higher melting temperatures than their unsaturated counterparts or incorporating cholesterol has been shown to help retain the encapsulated drug molecules when the liposome formulation is stored for long periods of time [65−68]. Protecting the vial from light and storing the formulation under an inert gas significantly reduces the risks of lipid oxidation as does adding an antioxidant, buffer, or chelating agents such as tocopherols, butyl hydroxyl toluene, or 2-(N-morpholino) ethanesulfonic acid, or ethylene diamine tetraacetic acid [59,69].

When liposomal formulations are not sufficiently stable at 4°C, the next best option is to freeze the liposomes formulations for storage at −20°C or −80°C. While chemical degradation of the lipids is significantly slower at these low temperatures, the process of freezing the formulations in itself is known to lead to rupture, fusion, and aggregation of liposomes [67,70,71]. Preventing physical degradation of the liposomes upon freezing often requires adding a cryoprotectant, such as various sugars or small molecules like glycerol or proline [70,72]. Studies of model systems have also shown that the freezing rate, freezing temperature, and freezing time all

impact the physical stability of the liposomes and therefore must be optimized in manufacturing processes [67,70,71]. In some cases, faster freezing rates and higher freezing temperatures (i.e., −25°C vs. −196°C) minimize aggregation or fusion of the vesicles and improve drug retention [71,73]. Meanwhile, other formulations are more stable if they are slowly cooled [74], as the slow freezing rate decreases the degree of supercooling and reduces the osmotic pressure exerted on the liposomes because there is sufficient time for the water to equilibrate across the bilayer as the formulation freezes [75]. In addition to the stability of the liposomal formulations, the stability of the container and closure systems at freezing temperatures must also be considered. For example, the physical properties of halobutyl elastomer stoppers commonly used to cap vials are compromised at or below $\approx -50°C$, which means that the container closure system and product sterility may be compromised if stored at or below this temperature [76]. It is also important to keep in mind that frozen drug products must be strictly maintained at the specified temperature throughout their shelf life, which significantly increases the storage and transport costs.

When there are no viable alternatives, lyophilization is the storage method of last resort. Lyophilization—or freeze drying—is a process used to remove water from liposomal formulations in which the formulation is first frozen, and then the water is removed by sublimation under reduced pressure at low temperatures [73,77]. Since hydrolysis and oxidation of lipids is accelerated in the presence of water, removing the water improves the chemical stability of the liposomal formulations; however, the freezing and drying processes significantly affect the physical stability of the liposomes. Also, lyophilization adds several levels of complexity in terms of manufacturing processes and regulatory compliance. Lyophilization is a time- and energy-consuming process that adds an additional unit operation, increases costs, and also must conform to regulatory requirements [77]. There are also strict regulatory requirements for the physical appearance of the dried liposome "cake" and for the reconstitution conditions that add complexity to the drug approval process [78]. Perhaps the biggest challenge in lyophilizing liposomes is that despite several decades of research described in several recent review articles [74,75,79–81], there are still no "rules of thumb" for the design and development of lyophilized liposomal formulations [75].

Much of the research and optimization of lyophilization of liposomal formulations has focused on finding suitable cryoprotectants to prevent rupture, aggregation, and fusion of the liposomes during the freezing step. Early studies showed that mono- and disaccharide sugars were more effective cryoprotectants for liposomes than larger sugar molecules [70,77,82], and much of the subsequent research has also focused on these excipients. Sugars such as lactose, maltose, and trehalose, have all been successfully used to prevent leakage of encapsulated molecules from freeze-dried liposomes, but their effectiveness highly depends on the lipid composition [75]. The mechanism by which these sugars protect liposomes also is not fully understood, but there are two primary hypotheses. The water replacement hypothesis suggests that sugars form stable hydrogen bonds with the lipid

headgroups without affecting the bilayer structure as the water is removed. This model suggests successful cryoprotectants should contain large numbers of hydrogen bonding groups and explains why molecules like sugars, amino acids, and proteins are able to retain the liposome structure and drug encapsulation once the water is removed [75,81]. The other proposed mechanism is the vitrification model that suggests concentration of the non-volatile cryoprotectants increases the viscosity of the aqueous phase as the water is removed, and this increase in viscosity helps prevent the liposomes from coming too close together and fusing or aggregating during drying [75,81]. The vitrification model suggests that glass transition temperature, as well as, the size of the cryoprotectant molecules are important to consider, as these factor ultimately determine the structure of the glassy matrix that surrounds and separates individual liposomes in the lyophilized cake. It is generally accepted that successful cryoprotectants work by both mechanisms [75,81,83]. While the proposed models and many other studies in literature provide some guidance on selecting molecules that will be good cryoprotectants for liposomes, selection of the optimal cryoprotectant still ultimately relies on empirical trial-and-error during the formulation process.

In addition to selecting the optimal cryoprotectant, there are a number of other lyophilization conditions that must be optimized including the cryoprotectant concentration, presence of other excipients, freezing rate and temperature, and long-term storage temperature of the lyophilized cake [74,83–85]. For example, increasing the cryoprotectant concentration generally improves liposome stability [86], but too high of a cryoprotectant concentration can exert a high osmotic pressure on the liposomes as they freeze leading to rupture [87]. As a means to mitigate these competing effects, studies by Izutsu et al. suggest that either incorporating the cryoprotectant on both sides on the membrane or pre-cooling the formulation can help stabilize liposomes by minimizing the osmotic pressure exerted on the vesicle as the formulation freezes and the cryoprotectant concentrates [73]. The optimal freezing rate has also been shown to depend on the lipid composition [71,77,88]. Comparative studies of lyophilized liposomes composed of unsaturated and saturated lipids suggested that rigid bilayers (i.e., membranes with a higher bending modulus) formed by saturated lipids were more vulnerable to fast cooling rates but retained more of the encapsulated drugs upon rehydration [88]. Even after lyophilization, the lipid composition and temperature used to store the dried cake can affect the long term stability of the formulation [85,89]. For example, Payton et al. showed that the lipid degradation rate in lyophilized formulations increased with the degree of lipid unsaturation as well as storage temperature at and above room temperature [89].

Given the challenges in finding appropriate lyophilization conditions for liposomes in addition to added complexity in manufacturing processes and regulatory compliance, it is perhaps not surprising that there are only a few lyophilized liposomal formulations on the market [4]. Of the 20 approved liposomal medicines as of 2019—including generic formulations of products whose patents have expired—only three are packaged and stored as lyophilized cakes [81]. AmBisome is one of the approved lyophilized products, and long-term stability studies by its producer, Gilead, show that

there can be substantial benefits to finding appropriate lyophilization conditions [76]. AmBisome is a liposomal formulation of amphotericin B, a highly hydrophobic drug used to treat serious fungal infections. Studies have shown that amphotericin B preferentially and stably binds to cholesterol, which significantly reduces the toxicity of the drug and likely helps retain the drug within the hydrophobic bilayer even if the liposomes break apart when the formulation is lyophilized and rehydrated [76]. Because of the strong drug binding to cholesterol combined with the novel temperature stability of the saturated long chain lipids used to form the liposomes, AmBisome is approved for storage up to 25°C in the United States for as long as 2 years, thereby eliminating the need for cold chain storage [76]. Moreover, long-term stability studies have shown that the liposome structure and drug encapsulation are stable for more than 10 years when AmBisome is stored at −40°C [76].

19.6 Summary

The production of useful liposomal drug products requires appropriate sizing, purification, sterilization, and storage practices that can be implemented on a commercial scale. The full manufacturing process must also yield liposomes that exhibit the originally-desired function and meet regulatory requirements. The effect of the applied process steps and their interaction on the physical stability of the liposomes as well as on the chemical stability of the comprising lipids and drug must be considered, which makes for a complex optimization problem. For example, adding cholesterol to the formulation increases the long-term stability of liposomes, which is desirable from economic and regulatory perspectives, but can also necessitate more energy-intensive sizing processes or result in larger liposomes. Alternatively, incorporating a cryoprotectant into liposome formulations (for storage under freezing temperatures or to support lyophilization) adds complexity to the purification step since one additional, desired component is present. Ultimately, the projected growth of liposome-based drug formulations in coming years demands continued progress on understanding and improving the manufacture of liposomes.

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