pharmaceutics

MDPI

Review

Physicochemical Characterization, Manufacturing, and Stability of Liposomal Drug Delivery Systems: From Formulation Design to Pharmaceutical Translation

Abdullah Abdulrahman Aljasser [1], Mohammad J. Akbar [1], Hamoud Alotaibi [2], Mona Al Hamod [2], Majed A. Majrashi [3], Hajar Alghamdi [4], Reem Alromaihi [5] and Saqar Alarifi [6,7,8,*]

[1] Department of Pharmaceutics, College of Pharmacy, Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam 31441, Saudi Arabia; aaaljaser@iau.edu.sa (A.A.A.); mjakbar@iau.edu.sa (M.J.A.)
[2] Department of Pharmaceutics, Faculty of Pharmacy, Northern Border University, Rafha 73213, Saudi Arabia; hamoud.alotaibi@nbu.edu.sa (H.A.); mona.alhumodh@nbu.edu.sa (M.A.H.)
[3] Bioengineering Institute, Health Sector, King Abdulaziz City for Science and Technology (KACST), Riyadh 11442, Saudi Arabia; mmajrashi@kacst.gov.sa
[4] Department of Pharmacy Practice, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin 39911, Saudi Arabia; hajarm@uhb.edu.sa
[5] Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraydah 51452, Saudi Arabia; r.alromaihi@qu.edu.sa
[6] Department of Pharmaceutical Sciences, College of Pharmacy, King Saud Bin Abdulaziz University for Health Sciences, Riyadh 11481, Saudi Arabia
[7] King Abdullah International Medical Research Centre, Ministry of National Guard—Health Affairs, Riyadh 11481, Saudi Arabia
[8] Ministry of National Guard—Health Affairs, Riyadh 11426, Saudi Arabia
* Correspondence: arifis@ksau-hs.edu.sa

Abstract

Liposomal drug delivery systems have evolved into clinically relevant platforms for delivering small molecules, biologics, and vaccines. Nevertheless, a persistent translational gap remains between promising laboratory-scale formulations and reproducible pharmaceutical products. This review provides a practical framework linking liposome formulation, physicochemical characterization, manufacturing, and stability to pharmaceutical translation. Particular emphasis is placed on relationships between critical process parameters and critical quality attributes, including vesicle size, polydispersity index, zeta potential, morphology, lamellarity, encapsulation efficiency, drug loading, and in vitro release behavior. Preparation and cargo-loading strategies are discussed across conventional research-scale methods and approaches with potential for scalable manufacturing. Microfluidic production of conventional liposomes is distinguished from the manufacture of structurally distinct RNA-loaded lipid nanoparticles. Purification strategies and analytical determination of encapsulation efficiency are examined, with attention to the distinction between separation techniques and quantitative analytical methods. Lamellarity is highlighted as a structural determinant of encapsulation capacity, membrane organization, and release behavior, with complementary assessment by cryo-TEM, ^{31}P-NMR, and small-angle X-ray scattering. Quality-control challenges associated with aggregation, fusion, cargo leakage, phospholipid hydrolysis, oxidation, and sterilization are also considered. Dry-state stabilization through lyophilization and spray drying is discussed for its potential to improve long-term stability and reduce cold-chain dependence. Ligand-targeted liposomes introduce additional requirements for surface characterization and biological evaluation, while approved products and investigational formulations illustrate achievements and limitations in clinical translation. Overall, this review emphasizes the

Academic Editor: Elisabete M. S. Castanheira

Received: 4 October 2026
Revised: 29 October 2026
Accepted: 29 October 2026
Published: 2 November 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

importance of establishing relationships between formulation composition, processing, structural organization, stability, and functional performance to support more reproducible development and informed pharmaceutical translation.

Keywords: liposomes; physicochemical characterization; microfluidic manufacturing; lamellarity; encapsulation efficiency; pharmaceutical stability; lyophilization; spray drying; lipid nanoparticles

1. Introduction

Liposomes remain one of the most established and influential nanocarrier systems in pharmaceutical science, representing a rare example of a platform that has progressed from fundamental biophysical observation to clinically relevant therapeutic application. Their importance lies not only in their long developmental history but also in their continuing adaptability across multiple drug delivery systems, including small molecules, biologics, vaccines, and nucleic acid-based systems. The modern scientific foundation of this field was established by Bangham and colleagues, who described the spontaneous formation of closed phospholipid lamellae in aqueous media, structures that later became recognized as liposomes [1]. Since this seminal discovery, liposomes have evolved from model membrane systems into highly versatile carriers capable of encapsulating both hydrophilic agents within their aqueous core and lipophilic compounds within the lipid bilayer, thereby offering substantial formulation flexibility [2,3]. The principal structural classifications of liposomes, including unilamellar, multilamellar, and multivesicular systems, are shown in Figure 1.

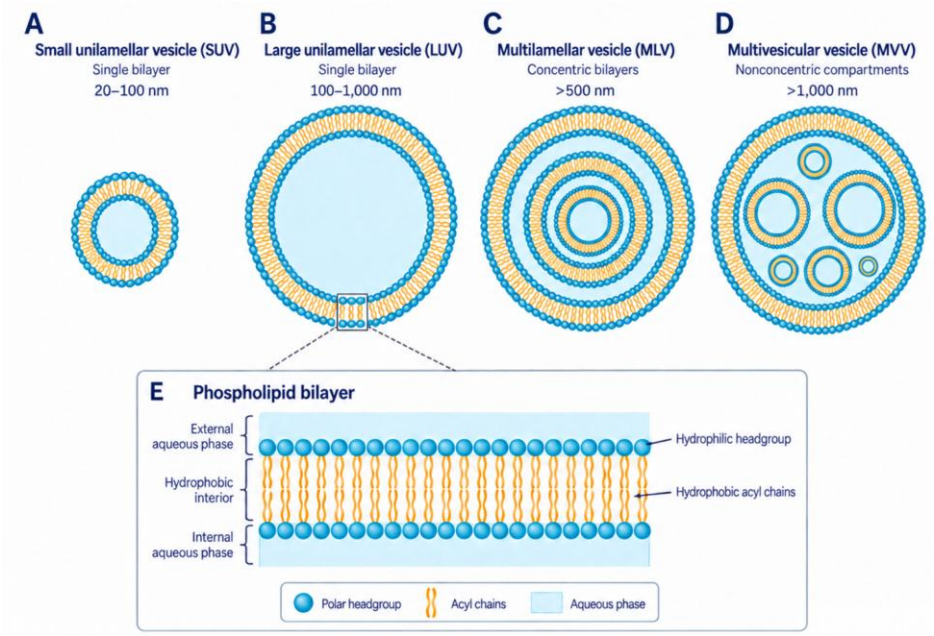


Figure 1. Schematic illustration of the major liposome types: small unilamellar vesicles (SUVs; 20–100 nm), large unilamellar vesicles (LUVs; 100–1000 nm), multilamellar vesicles (MLVs; >500 nm), and multivesicular vesicles (MVVs; >1000 nm). SUVs and LUVs consist of a single phospholipid bilayer enclosing an aqueous core and differ primarily in their size. MLVs exhibit multiple concentric bilayers, whereas MVVs contain multiple internal aqueous compartments within a single outer bilayer. The inset shows the organization of the phospholipid bilayer, with hydrophilic head groups oriented toward the aqueous environment and hydrophobic acyl chains forming the core of the bilayer.

https://doi.org/10.3390/pharmaceutics18111412

At the molecular level, liposome formation is driven by the self-assembly of amphiphilic lipids in aqueous environments. One of the most widely used theoretical concepts for interpreting this behavior is the critical packing parameter (CPP), which links the lipid molecular geometry to the supramolecular structures that are likely to form [4]. CPP is expressed as Equation (1):

$$CPP = \frac{v}{a_0 \times l} \quad (1)$$

Equation (1): critical packing parameter equation where v is the hydrocarbon chain volume, a_0 is the effective polar headgroup area, and l is the critical hydrophobic chain length.

In general, lipids with geometries that favour near-cylindrical packing are more likely to assemble into bilayers, which can then close to form vesicular structures, such as liposomes. In contrast, other geometric arrangements may favor micellar or non-lamellar phases, as shown in Figure 2. However, liposome formation is not solely dictated by molecular geometry. Hydration conditions, ionic strength, temperature, lipid purity, and the presence of membrane additives all influence bilayer curvature, lamellarity, membrane order, and colloidal stability. This means that liposome assembly must be understood as the outcome of both thermodynamic tendencies and formulation conditions [2,4].

The functional properties of liposomes are strongly dependent on their lipid composition. Phospholipids provide the structural framework of the vesicles, whereas cholesterol is frequently incorporated to regulate membrane fluidity, improve bilayer packing, reduce permeability, and enhance the retention of encapsulated cargo. More sophisticated formulations may also include PEGylated lipids to prolong circulation, charged lipids to alter interfacial behavior, and helper lipids that support loading efficiency and intracellular delivery. Beyond conventional lipid additives, modification of membrane composition can directly alter bilayer biophysical behavior and cellular interactions. For example, the incorporation of α-linolenic acid into phospholipid liposomes increased membrane fluidity and promoted site-restricted fusion with the cell plasma membrane, illustrating how lipid composition can influence not only vesicle structure but also the mechanism of cellular delivery [5]. These compositional refinements have substantially expanded the pharmaceutical scope of liposomes and related lipid-based carriers. Simultaneously, it is crucial to differentiate classical liposomes from the more recently developed lipid nanoparticles employed in mRNA delivery. Although these systems are historically related and share certain design principles, they are not structurally identical and should not be considered interchangeable in scientific discourse [6,7].

Despite decades of progress, the translation of liposomal systems from laboratory formulations to reproducible pharmaceutical products remains a challenge. Liposomes are highly sensitive colloidal assemblies, and even subtle changes in their physicochemical properties can markedly affect their behavior. Parameters such as vesicle size, size distribution, lamellarity, surface charge, membrane composition, lipid oxidation, and phase behavior can influence storage stability, cargo leakage, interaction with biological fluids, biodistribution, and therapeutic outcomes [8]. Consequently, promising biological activity alone is insufficient for successful development unless it is supported by rigorous physicochemical characterization and a clear understanding of the relationship between formulation attributes and product performance [2,7–9]. This challenge helps explain why the literature contains a large number of liposomal formulations with encouraging preclinical findings, whereas only a limited subset achieves meaningful clinical translation [3,10,11].

This translational gap underscores the importance of quality-oriented liposome characterization during formulation development. In practice, liposome research sometimes places disproportionate emphasis on therapeutic applications while giving insufficient

attention to the analytical rigor needed to define formulation quality. Physicochemical characterization is fundamental not only for descriptive purposes but also for ensuring formulation rationality, reproducibility, scalability, and eventual pharmaceutical applicability. Attributes such as vesicle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency are particularly important because they directly affect structural integrity, physical stability, release behavior, and biological interactions. Therefore, a meaningful interpretation of these parameters is essential not only for comparing formulations but also for understanding how liposomal systems may be optimized for distinct therapeutic purposes and routes of administration [2,7].

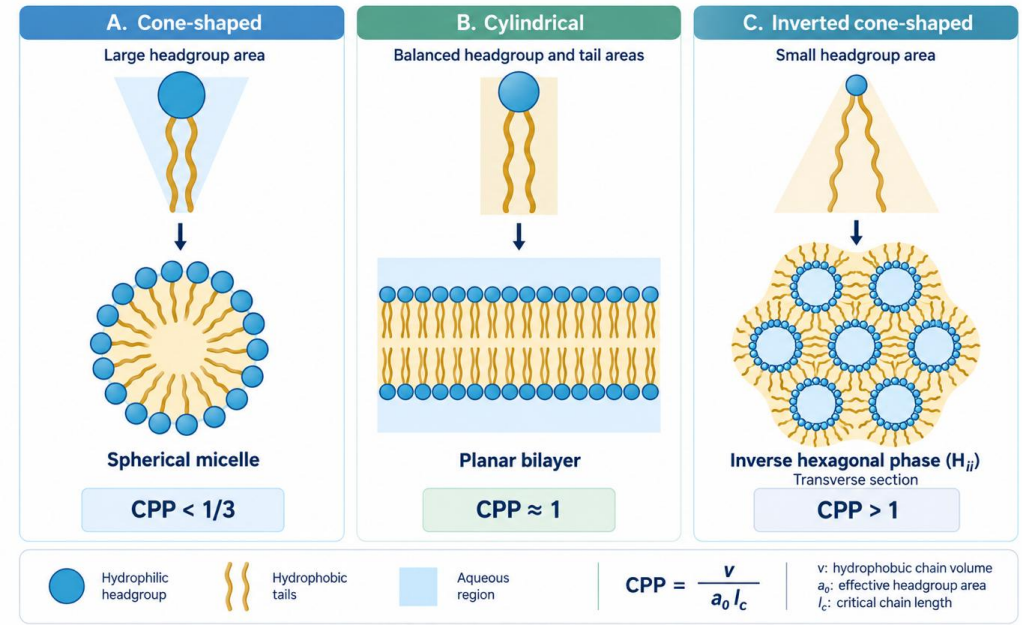


Figure 2. Critical packing parameter (CPP) and lipid self-assembly. Schematic illustration of how lipid molecular geometry governs the formation of supramolecular structures. (**A**) Cone-shaped lipids with relatively large head groups and smaller hydrophobic tails favor micelle formation (CPP < 1/3). (**B**) Cylindrical lipids with balanced headgroup and tail dimensions favor the formation of planar bilayers and liposomes (CPP ≈ 1). (**C**) Inverted cone-shaped lipids with relatively small headgroups and large hydrophobic tails promote the formation of inverted non-lamellar structures, such as the inverse hexagonal phase (H_{II}) (CPP > 1). The critical packing parameter (CPP = $v/a_0 \times l_c$) relates the hydrophobic tail volume (v), optimal headgroup area (a_0), and critical chain length (l_c), providing a theoretical framework for predicting the lipid self-assembly behavior.

Accordingly, the primary objective of this review is to connect the fundamental principles of lipid self-assembly with the practical requirements for developing functional liposomal drug delivery systems. Although the literature extensively describes the therapeutic promise of nanomedicine, there remains a clear need for a consolidated and application-oriented framework that emphasizes the physicochemical characterization required for successful liposome formulation. In this context, the present review systematically examines key characterization parameters, particularly vesicle size, polydispersity index, surface charge, and encapsulation efficiency, and discusses how these attributes govern structural integrity, physical stability, and in vitro release behavior. In addition, it highlights how the rational manipulation of these properties can be used to tailor liposomal systems for different therapeutic goals and routes of administration. Ultimately, this review aims to provide a practical and scientifically grounded reference for formulation scientists and researchers working to advance lipid-based therapeutics from bench-scale design to pharmaceutical applications.

2. Liposome Preparation and Manufacturing Strategies

The transition of liposomes from laboratory constructs to clinically relevant drug delivery systems has been largely driven by advances in formulation technology. Although all liposome preparation methods rely on the self-assembly of amphiphilic lipids in an aqueous environment, the manner in which this assembly is initiated strongly influences the vesicle size, lamellarity, encapsulation efficiency, and batch reproducibility. Consequently, the formulation strategy is not simply a technical detail; it is a crucial determinant of product quality and translational potential. In contemporary liposome development, there is an increasing emphasis on methods that support process control, scalability, and manufacturing reproducibility, particularly for formulations intended for clinical or industrial use.

2.1. Conventional Research-Scale Preparation Methods

Among conventional approaches, thin-film hydration remains the most widely used method in small-scale liposome research because of its simplicity, flexibility, and compatibility with a wide range of lipid compositions. In this method, lipids are first dissolved in an organic solvent, commonly chloroform or a chloroform–methanol mixture. The solvent is then removed under reduced pressure to produce a thin lipid film along the wall of the container. When this film is hydrated with an aqueous phase, typically at a temperature above the phase transition temperature of the principal lipid components, the dried lipids swell and self-assemble into vesicular structures, usually multilamellar vesicles [12,13].

The major advantage of thin-film hydration is its practicality. It is experimentally straightforward, requires relatively simple equipment, and can be adapted for both hydrophilic and lipophilic drug loading. For this reason, it remains highly valuable during early-stage formulation screening and proof-of-concept studies. Despite its utility, the method has notable limitations. The vesicles produced tend to be large, multilamellar, and heterogeneous, with a wide range of sizes. This variability often renders them unsuitable for direct therapeutic use, particularly when systemic delivery is required [12,14].

Accordingly, liposomes produced by thin-film hydration frequently require a subsequent post-formation size-reduction step. Extrusion is one of the most commonly used approaches. In extrusion, preformed liposomes are passed through polycarbonate membranes with defined pore sizes, allowing a reduction in vesicle diameter and narrowing of particle size distribution. Importantly, extrusion should be regarded primarily as a post-formation size-reduction and homogenization technique, rather than a primary formulation method. It does not generate liposomes de novo but instead refines already formed vesicles to improve size uniformity and, in many cases, promotes the production of more homogeneous unilamellar populations. Compared with probe sonication, extrusion is generally considered less destructive and less likely to induce metal contamination or severe local overheating, although lipid loss and filter-related material retention may still occur [15]. Despite its utility, the combination of thin-film hydration followed by extrusion remains labour-intensive and difficult to translate directly into large-scale manufacturing because of batch variability and limited process continuity.

Other established preparation methods provide particular advantages depending on the nature of the therapeutic cargo. Reverse-phase evaporation, for example, involves the formation of a water-in-organic-solvent emulsion, followed by the gradual removal of the organic phase, producing vesicles with a relatively large internal aqueous volume and high encapsulation efficiency for hydrophilic compounds. This is an important advantage when proteins, nucleic acids, or other water-soluble molecules are incorporated. However, the requirement for organic solvents and emulsification exposes the formulation and encapsulated cargo to conditions that may be undesirable for sensitive biomolecules and

introduces an additional solvent-removal requirement [16]. Detergent-removal methods provide another approach in which lipids are initially solubilized as mixed lipid–detergent micelles and subsequently reorganize into vesicles as the detergent is progressively removed, for example, by dialysis, dilution, chromatography, or adsorption. These methods can produce relatively homogeneous unilamellar vesicles and are particularly useful when membrane-associated proteins are incorporated into the bilayer. Nevertheless, they are comparatively time-consuming, and complete removal of residual detergent can be technically challenging [17].

In addition to these established approaches, dual asymmetric centrifugation (DAC), also referred to as dual centrifugation (DC), has emerged as an alternative technique for rapid, small-batch liposome preparation. Unlike conventional centrifugation, DAC combines rotation around a primary axis with an additional rotation of the sample container around a secondary axis, generating intensive mixing and shear forces within a closed vial. Typically, concentrated lipid mixtures are homogenized with an aqueous phase to form a viscous vesicular phospholipid gel (VPG), which can subsequently be diluted to obtain a liposomal dispersion [18]. Massing et al. first demonstrated the applicability of DAC to liposome preparation, producing vesicles with a mean diameter of approximately 60 nm and a calcein entrapment efficiency of 56% under optimized conditions [18]. Subsequently, Hirsch et al. extended this technique to siRNA-loaded liposomes, reporting entrapment efficiencies ranging from 43% to 81%, depending on batch size, encapsulated compound, and liposome composition [19]. More recently, Koehler et al. demonstrated the suitability of dual centrifugation for rapid, parallel formulation screening, including the preparation of multiple lipid compositions without prior formation of a molecularly mixed lipid film [20]. Gleue et al. further applied this approach to the screening and development of pH-responsive liposomal formulations [21]. These findings highlight the potential of DAC for efficient laboratory-scale preparation, encapsulation of hydrophilic compounds, and formulation optimization. Nevertheless, liposome characteristics remain sensitive to processing parameters, particularly lipid concentration, rotational speed, homogenization time, and mixing aids, while the application of liposomes to industrial-scale manufacturing requires further evaluation.

Despite their continued importance in laboratory research, many conventional solvent- and detergent-based preparation methods have several limitations from a translational perspective. Liposome formation frequently occurs under bulk-processing conditions, in which local solvent composition, lipid concentration, temperature, and mixing conditions may not be uniformly controlled. Consequently, vesicle size, size distribution, lamellarity, and encapsulation efficiency may vary between preparations. Furthermore, many conventional techniques require additional processing steps, such as solvent or detergent removal, sonication, homogenization, or extrusion, increasing processing time and providing additional opportunities for material loss and batch-to-batch variability. These limitations become increasingly important during scale-up, when reproducing the same mixing and self-assembly conditions achieved at the laboratory scale can be difficult. Such challenges have encouraged the development of manufacturing technologies in which nanoparticle self-assembly can be controlled more precisely [22,23].

2.2. Scalable and Translational Manufacturing Approaches

To address the limitations of conventional batch methods, increasing attention has shifted toward solvent-displacement and continuous-flow approaches that offer improved reproducibility and greater scalability. One of the simplest among these is ethanol injection. In this method, lipids dissolved in ethanol are rapidly introduced into an aqueous phase, and the sudden reduction in solvent quality drives spontaneous vesicle formation. Ethanol injection is operationally simpler than thin-film hydration and avoids the

need to first generate and rehydrate a dried lipid film. It is also more amenable to scale-up and can be performed with fewer manual steps. However, conventional ethanol injection may produce liposomes with relatively broad size distributions, and downstream removal of residual ethanol remains an important consideration, particularly for pharmaceutical applications [24].

The proliposome approach is a distinct formulation strategy that addresses some of the storage limitations of aqueous liposome dispersions. Proliposomes are dry, free-flowing precursor systems, typically powders or granules, in which phospholipids and drugs are deposited onto water-soluble carrier materials, such as sugars or sorbitol. They do not constitute liposomes themselves; rather, they generate liposomal vesicles upon contact with an aqueous medium through hydration of the lipid-coated carrier surface [25,26]. The principal rationale for this approach is its improved stability. Conventional aqueous liposomal dispersions are susceptible to aggregation, fusion, phospholipid hydrolysis, and oxidation during storage, all of which may compromise the vesicle integrity and drug retention. Maintaining lipid components in a dry state until hydration can reduce these degradation processes and facilitate their incorporation into solid dosage forms. Common preparation methods include the slurry technique, spray drying, freeze drying, and fluid-bed coating, each of which produces solid intermediates with different particle characteristics and reconstitution behavior [25]. Although proliposomes have been investigated for several administration routes, they are particularly attractive for oral delivery because of their compatibility with solid dosage form manufacturing and potential for improved storage stability. Nevertheless, their development requires optimization of both the dry precursor and the subsequent hydration process, because vesicle size, homogeneity, and drug-loading characteristics after reconstitution cannot be assumed to be equivalent to those obtained using conventional liposome preparation [26,27].

Microfluidic mixing represents a significant advance toward the controlled and reproducible manufacturing of lipid-based nanocarriers. In this approach, lipid-containing organic and aqueous phases are combined under controlled flow conditions, allowing solvent exchange and lipid self-assembly. Microfluidic configurations, including hydrodynamic-flow-focusing and staggered-herringbone mixers, enable adjustments to processing parameters such as total flow rate (TFR), flow-rate ratio (FRR), lipid concentration, and solvent composition, which can influence particle size, size distribution, and formulation reproducibility [22,23]. Although these manufacturing principles are applicable to different lipid-based nanocarriers, the mechanisms of particle formation and resulting structures differ between conventional liposomes and RNA-loaded lipid nanoparticles (RNA-loaded LNPs). These applications are discussed separately in the following subsections.

2.3. Advances in Microfluidic Manufacturing of RNA-Loaded Lipid Nanoparticles

Microfluidic technology has played an important role in advancing the manufacturing of RNA–LNPs by enabling controlled and reproducible nanoparticle formation. Although LNPs share several lipid components with conventional liposomes, they differ in their structural organization and mechanisms of cargo incorporation. Conventional liposomes consist of one or more phospholipid bilayers enclosing aqueous compartments, whereas RNA-loaded LNPs generally exhibit a more complex internal lipid–RNA organization rather than the well-defined aqueous core characteristic of classical liposomes [28]. RNA–LNP formulations typically comprise four major lipid components: an ionizable lipid, a helper phospholipid, cholesterol, and a PEGylated lipid. Under acidic formulation conditions, ionisable lipids become protonated and positively charged, facilitating electrostatic interactions with negatively charged RNA and promoting its incorporation during nanoparticle assembly [29].

In microfluidic manufacturing, an ethanolic lipid solution is rapidly mixed with an acidic aqueous phase containing RNA. The resulting solvent exchange promotes lipid self-assembly, whereas interactions between protonated ionizable lipids and RNA facilitate nanoparticle formation and encapsulation. Critical processing variables, including TFR, FRR, lipid concentration, solvent composition, aqueous-phase pH, and mixer geometry, can influence particle size, size distribution, encapsulation efficiency, and structural organization. By providing greater control over mixing conditions, appropriately optimized microfluidic systems can improve manufacturing consistency and reduce batch-to-batch variability compared with conventional bulk-mixing approaches [30,31].

The pharmaceutical significance of controlled mixing became particularly evident during the development of mRNA vaccines for SARS-CoV-2. For example, the LNPs used in the Pfizer–BioNTech BNT162b2/COMIRNATY vaccine were manufactured using impinging-jet mixing systems operated in parallel to support large-scale production. Although these turbulent mixing systems differ technically from conventional chip-based microfluidic devices, both approaches rely on the rapid and controlled mixing of lipid and aqueous streams to regulate nanoparticle assembly [32,33]. Furthermore, the parallelization of microfluidic platforms has demonstrated the potential to increase RNA–LNP production throughput while maintaining particle characteristics and biological activity [34]. Nevertheless, challenges involving equipment requirements, process optimization, residual solvent removal, downstream purification, and industrial scale-up remain important considerations. These developments highlight the translational potential of controlled-flow manufacturing while emphasizing that RNA-loaded LNPs are structurally distinct from conventional liposomes.

2.4. Microfluidic Production of Conventional Liposomes

Beyond its application to RNA-loaded LNPs, microfluidic technology has been extensively investigated for the controlled and scalable production of conventional liposomal vesicles. Unlike RNA–LNP manufacturing, where lipid–nucleic acid interactions contribute substantially to nanoparticle formation, conventional liposome production primarily relies on the self-assembly of amphiphilic lipids into bilayer structures following controlled solvent exchange. Microfluidic hydrodynamic focusing is among the earliest approaches used for this purpose, in which a lipid-containing organic stream is focused by adjacent aqueous streams to regulate solvent diffusion and vesicle formation. Early studies by Jahn et al. demonstrated the feasibility of producing size-controlled liposomes using microfluidic flow conditions [35,36]. Subsequent investigations by Kastner et al. demonstrated the importance of TFR and FRR in controlling liposome characteristics, supporting the application of microfluidic manufacturing for reproducible vesicle preparation [37].

Further developments have expanded the pharmaceutical applicability of microfluidic liposome manufacturing beyond particle-size control. Kastner et al. demonstrated the preparation of propofol-loaded liposomes using a chaotic-advection micromixer, achieving drug incorporation of approximately 41 mol% and tunable vesicle sizes ranging from 50 to 450 nm [38]. Similarly, Forbes et al. developed a microfluidic manufacturing approach for protein-loaded liposomes that incorporated in-line purification and at-line particle-size monitoring [39]. This integrated strategy highlighted the potential for combining vesicle formation with downstream processing and analytical monitoring to improve manufacturing efficiency.

A particularly important advancement was demonstrated by Webb et al., who investigated the scale-independent microfluidic production of protein-loaded liposomes using staggered-herringbone and toroidal mixing configurations [40]. Their study demonstrated controlled liposome manufacturing from small laboratory-scale preparations to

processing rates of 200 mL/min while maintaining defined physicochemical characteristics, including vesicle size, polydispersity, protein loading, and release behavior. These findings provided experimental evidence supporting the feasibility of translating microfluidic liposome manufacturing toward good manufacturing practice (GMP)-relevant production scales. In addition, Roces et al. demonstrated the continuous microfluidic manufacturing of PEGylated liposomes followed by remote loading of doxorubicin, producing vesicles with sizes of approximately 80–100 nm, polydispersity indices below 0.2, and encapsulation efficiencies of at least 90% [41]. These studies highlight the potential of combining controlled vesicle fabrication with established active-loading strategies to manufacture pharmaceutically relevant liposomal formulations.

Nevertheless, the relationship between manufacturing conditions and liposome performance extends beyond conventional particle-size measurements. Solvent selection, lipid composition, FRR, and loading conditions can influence vesicle characteristics and formulation behavior. More recently, Lindsay et al. investigated the influence of solvent selection during microfluidic manufacturing on the in vivo performance of liposomal doxorubicin [42]. Although formulations produced using different solvents exhibited broadly comparable vesicle sizes, polydispersity, and encapsulation efficiencies, differences in drug biodistribution were observed, emphasizing that conventional physicochemical attributes alone may not fully predict biological performance. Despite continued advances, limitations associated with equipment requirements, channel fouling, solvent removal, downstream purification, and process validation remain important considerations. Overall, these investigations demonstrate the potential of microfluidic manufacturing to improve the reproducibility and scalability of conventional liposomes while highlighting the importance of understanding the relationships between process parameters, critical quality attributes (CQAs), and pharmaceutical performance. The principal preparation and processing approaches discussed above are compared in Table 1, while Figure 3 schematically illustrates thin-film hydration, ethanol injection, and microfluidic mixing.

Table 1. Comparative Overview of Liposome Formation and Post-Formation Processing Methods.

Approach	Classification	Principle	Main Advantages	Main Limitations	References
Thin-film hydration	Primary formulation method	Hydration of a dry lipid film to form liposomal vesicles	Simple, versatile, and widely used in laboratory research	Frequently produces heterogeneous and multilamellar vesicles; often requires further size reduction; limited direct scalability	[13]
Ethanol injection		Solvent displacement following injection of an ethanolic lipid solution into an aqueous phase	Simple preparation process; avoids dry-film formation; relatively amenable to scale-up	Residual ethanol requires removal; vesicle size and size distribution depend strongly on mixing conditions	[24]
Reverse-phase evaporation		Formation of a water-in-organic-solvent emulsion followed by gradual solvent evaporation	Relatively high aqueous-volume entrapment; suitable for encapsulating hydrophilic compounds	Organic solvent exposure; additional solvent-removal steps; potentially unsuitable for sensitive therapeutic cargo	[16]
Detergent removal		Formation of liposomal vesicles from mixed lipid–detergent micelles through progressive detergent removal	Can produce relatively homogeneous unilamellar vesicles; useful for incorporating membrane-associated proteins	Time-consuming; complete detergent removal may be challenging; residual detergent may compromise formulation quality	[17]
Dual asymmetric centrifugation		High-shear homogenization of concentrated lipid mixtures through dual-axis rotation, producing a vesicular phospholipid gel that is subsequently diluted	Rapid small-batch preparation; closed-vial processing; efficient encapsulation of hydrophilic compounds; suitable for parallel formulation screening	Specialized equipment required; vesicle characteristics depend on processing parameters; limited evidence supporting industrial-scale manufacturing	[18–21]
Proliposome approach	Dry precursor formulation strategy	Formation of liposomal vesicles upon hydration of a dry lipid-containing precursor	Potentially improved storage stability; compatible with solid dosage forms; reduced dependence on aqueous storage	Vesicle characteristics depend on reconstitution conditions; additional formulation optimization and characterization required	[25,26]
Extrusion	Post-formation size-reduction technique	Passage of preformed liposomes through membranes with defined pore sizes	Reduces vesicle diameter and narrows size distribution; improves vesicle homogeneity	Additional processing step; potential lipid and material loss; limited throughput with conventional setups; does not independently generate liposomes	[15]
Microfluidic mixing	Controlled-flow primary formulation method	Controlled mixing of lipid-containing organic and aqueous phases to induce solvent exchange and vesicle self-assembly	Improved control over vesicle size and size distribution; enhanced process reproducibility under optimized conditions; potential for continuous and scalable manufacturing	Requires specialized equipment and process optimization; solvent removal and downstream purification may be necessary; throughput and scalability depend on mixer configuration	[33,35,37]

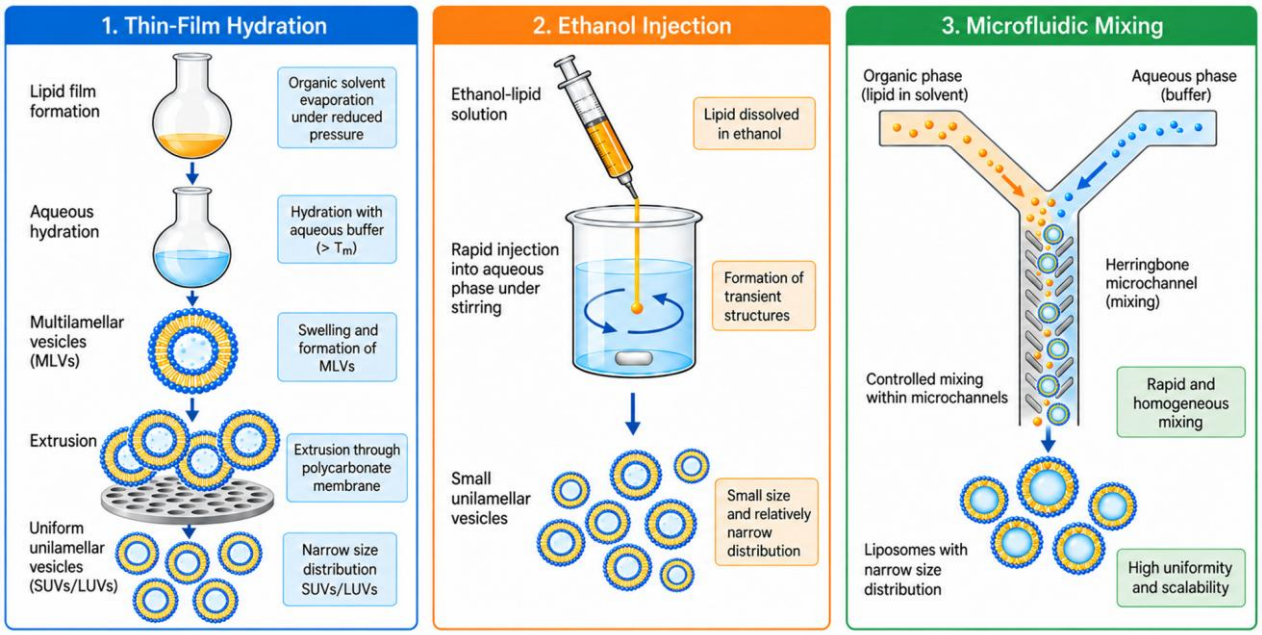


Figure 3. Comparison of liposome formulation methods. Schematic overview of three commonly used liposome preparation techniques. In thin-film hydration, lipids are dissolved in an organic solvent, evaporated to form a thin film, and subsequently hydrated to produce multilamellar vesicles (MLVs), which are then passed through a porous polycarbonate membrane during extrusion to reduce vesicle size and obtain more uniform unilamellar vesicles (SUVs/LUVs). In the ethanol injection method, a lipid solution in ethanol is rapidly introduced into an aqueous phase, leading to spontaneous vesicle formation through solvent displacement. In microfluidic mixing, organic and aqueous phases are combined within microchannels under controlled flow conditions, enabling the rapid and reproducible self-assembly of liposomes with narrow size distributions. These approaches differ in terms of process control, scalability, and particle uniformity, with microfluidic systems offering the highest level of reproducibility and tunability.

3. Cargo-Loading Strategies: Passive and Active (Remote) Loading

The therapeutic performance of liposomal systems depends not only on vesicle composition and structure but also on the efficiency with which the cargo is incorporated and retained until delivery to the target site. Drug-loading strategies are generally classified as passive or active, depending on whether the compound is entrapped during vesicle formation or introduced into pre-formed liposomes under a driving gradient [43,44].

3.1. Passive-Loading Strategy

Passive loading is the simplest and most widely used strategy during liposome preparation. Lipophilic molecules are typically dissolved in the organic lipid phase and partition into the forming bilayer, whereas hydrophilic compounds are dissolved in the aqueous hydration medium and become entrapped within the internal aqueous core during vesicle closure. Despite its convenience, passive loading is often limited for hydrophilic drugs because encapsulation depends strongly on the trapped aqueous volume, which is typically low in conventional liposomal systems. Consequently, passive loading often results in low encapsulation efficiencies unless high lipid concentrations or specialized formulations are employed [45–47].

3.2. Active-Loading Strategy

Active or remote loading represents one of the most pharmaceutically important advances in liposome formulation, particularly for weakly basic or amphipathic drugs. In this approach, the drug is loaded into pre-formed liposomes by exploiting a transmembrane gradient, most commonly a pH gradient or an ammonium sulfate gradient. The uncharged form of the drug diffuses across the lipid bilayer, after which it becomes protonated or forms a poorly permeable complex within the intraliposomal environment. This process results in efficient intravesicular trapping, often accompanied by precipitation or gel-like drug condensation, thereby markedly improving encapsulation efficiency, retention, and formulation stability.

Compared with passive loading, remote loading offers major pharmaceutical advantages, including higher drug-to-lipid ratios, reduced premature leakage, and improved shelf-life stability. These benefits have made it a key enabling strategy in clinically translated liposomal products, most notably in liposomal doxorubicin formulations such as Doxil. Accordingly, remote loading should be regarded not merely as a method to improve encapsulation efficiency but as a central formulation strategy that has significantly advanced the clinical utility of liposomal drug delivery systems [24,48,49].

Overall, the selection of an appropriate cargo-loading strategy should be guided by the physicochemical properties of the therapeutic compound, the lipid composition, and the intended pharmaceutical application. Passive loading offers a straightforward approach for incorporating therapeutic agents during vesicle formation, whereas active loading enables efficient drug incorporation into preformed liposomes through transmembrane gradients. Both strategies influence encapsulation efficiency, drug retention, and release behavior, highlighting the importance of selecting and optimizing loading conditions to achieve the desired pharmaceutical performance.

4. Critical Physicochemical Attributes and Analytical Characterization

Physicochemical characterization is central to liposome development because the therapeutic performance of a formulation depends not only on its composition but also on how its structural and interfacial properties are measured, interpreted, and controlled. In practice, liposomes that appear similar at the level of lipid composition may behave very differently if they differ in size distribution, surface charge, lamellarity, drug entrapment, or release profile. For this reason, characterization should not be treated as a routine checklist performed after formulation but as an integrated analytical framework that links composition and process variables to product quality, stability, and biological function. Recent quality-by-design discussions in the liposome field have reinforced this point by treating parameters such as size, polydispersity, zeta potential, encapsulation efficiency, and release behavior as CQAs that must be defined early and controlled throughout development [50,51].

4.1. Relationships Between Critical Process Parameters and Critical Quality Attributes

The physicochemical characteristics of liposomal formulations are influenced not only by lipid composition and drug properties but also by the manufacturing conditions employed during vesicle formation and subsequent processing. Within the quality-by-design framework, critical quality attributes (CQAs) are physical, chemical, biological, or microbiological characteristics that must be maintained within appropriate limits to ensure the desired product quality. Critical process parameters, in contrast, are manufacturing variables whose variability can affect CQAs and therefore require appropriate monitoring or control. Establishing relationships between process parameters and CQAs is

essential for developing reproducible manufacturing processes and ensuring consistent pharmaceutical performance [52,53].

The influence of manufacturing conditions on liposome properties varies according to the preparation technique. In conventional thin-film hydration, hydration temperature, duration, and agitation conditions may affect lipid film dispersion, vesicle formation, lamellarity, and particle size distribution. Subsequent extrusion introduces additional variables, including membrane pore size, processing temperature, applied pressure, and the number of extrusion passes, which can influence vesicle size, polydispersity, and lipid recovery. Similarly, reverse-phase evaporation and detergent-removal techniques require control over emulsification or detergent-removal conditions, respectively, because these parameters can affect vesicle formation, encapsulation efficiency, and residual solvent or detergent content. For ethanol injection, injection conditions, phase ratios, and mixing intensity influence solvent exchange and liposome assembly, potentially affecting vesicle size, size distribution, and drug incorporation [54,55].

Microfluidic manufacturing offers opportunities to establish more systematic relationships between processing variables and liposomal CQAs. In particular, TFR, FRR, lipid concentration, solvent composition, and mixer geometry influence the mixing environment and subsequent vesicle formation. Forbes et al. investigated the influence of microfluidic operating parameters and formulation composition on the integrated production and purification of protein-loaded liposomes. Their findings highlight the importance of evaluating manufacturing conditions alongside particle size, polydispersity, and cargo encapsulation rather than treating these attributes as independent formulation outcomes [39].

Beyond vesicle formation, downstream processing and cargo loading introduce additional variables that may influence final product quality. For example, during active drug loading, the transmembrane pH or ion gradient, incubation temperature, loading duration, and drug-to-lipid ratio can affect encapsulation efficiency, drug retention, and release behavior. Likewise, purification, solvent removal, and dry-state processing may influence vesicle integrity, particle size distribution, and chemical stability. Consequently, the identification of relevant critical process parameters should consider the entire manufacturing process rather than individual preparation steps alone [52,53].

Table 2 summarizes the principal manufacturing variables associated with the fabrication methods discussed in Section 2 and their potential relationships with liposomal CQAs. Importantly, the listed parameters should be regarded as candidates for criticality assessment, since their actual criticality depends on the formulation, intended application, manufacturing scale, and experimentally established effects on product quality. Establishing and evaluating these relationships provides a foundation for process optimization, scale-up, and pharmaceutical translation [52,53]. The application of statistical experimental design and emerging data-driven approaches to liposome formulation optimization is discussed in the following subsection.

Table 2. Relationships Between Manufacturing Process Parameters and Liposomal CQAs.

Fabrication Method	Potential Critical Process Parameters	CQAs Potentially Affected	Mechanistic Relationship	Reference
Thin-film hydration	Hydration temperature, time, agitation rate	Vesicle size, PDI, lamellarity, EE	Hydration conditions affect lipid swelling, bilayer detachment, and vesicle formation	[54,56]
Extrusion	Membrane pore size, pressure, temperature, number of passes	Vesicle size, PDI, lamellarity, lipid recovery	Passage through membranes modifies vesicle dimensions and may alter bilayer organization	[16,55]
Reverse-phase evaporation	Emulsification intensity, aqueous-to-organic ratio, solvent-removal conditions	Vesicle size, EE, morphology, residual solvent	Emulsion formation and solvent evaporation influence aqueous entrapment and vesicle assembly	[54,55]
Detergent removal	Detergent-to-lipid ratio, detergent-removal rate, temperature	Vesicle size, PDI, lamellarity, residual detergent	Detergent depletion controls the transition from mixed micelles to bilayer vesicles	[17]
Dual asymmetric centrifugation	Lipid concentration, rotational speed, homogenization time, mixing aids	Vesicle size, PDI, EE, formulation reproducibility	Dual-axis rotation generates intensive mixing and shear forces, promoting vesicle formation and drug encapsulation	[18–21]
Ethanol injection	Injection rate, aqueous-to-organic ratio, mixing intensity, temperature	Vesicle size, PDI, EE, residual ethanol	Solvent dilution and mixing kinetics govern lipid self-assembly	[24]
Proliposomes	Drying temperature, drying conditions, coating or carrier-loading conditions	Drug retention, residual moisture, reconstituted vesicle size and PDI	Drying and carrier properties affect lipid integrity and subsequent vesicle formation	[25,26]
Microfluidic mixing	TFR, FRR, lipid concentration, solvent composition, mixer geometry	Vesicle size, PDI, EE, morphology, reproducibility	Controlled solvent exchange and mixing influence vesicle nucleation and growth	[37,38,42]

4.2. Design of Experiments and AI-Assisted Formulation Optimization

The development of liposomal formulations involves the simultaneous optimization of multiple formulation and process variables that influence CQAs. Design of experiments (DoE) provides a systematic statistical framework for evaluating these relationships while reducing reliance on traditional one-factor-at-a-time approaches. Commonly employed strategies include factorial designs, response surface methodology, and Box–Behnken and central composite designs, which allow the assessment of individual factors, interactions, and nonlinear effects. In liposome development, these approaches have been applied to optimize lipid composition, cholesterol content, processing conditions, vesicle size, encapsulation efficiency, and drug release behavior. For example, Kastner et al. employed DoE to investigate the effects of total flow rate and flow-rate ratio during microfluidic liposome manufacturing, demonstrating the importance of processing conditions in controlling vesicle characteristics [37]. Similarly, Khute and Jangde applied a Box–Behnken design to optimize venlafaxine-loaded liposomes by evaluating formulation variables against particle size, encapsulation efficiency, and drug release responses [57].

More recently, artificial intelligence (AI), particularly machine learning (ML), has emerged as a complementary approach for exploring complex formulation–process relationships that may be difficult to describe using conventional statistical models alone. Unlike traditional DoE approaches, which generally rely on predefined experimental designs and fitted response models, ML algorithms can identify nonlinear patterns within larger multidimensional datasets and support the prediction of formulation outcomes. Eugster et al. (2024) demonstrated this potential by developing ML models using a dataset comprising more than 1300 microfluidically produced liposome formulations [58]. Their models predicted liposome formation, particle size, and relevant manufacturing parameters, with experimental validation supporting their applicability to formulation development. Subsequently, Buttitta et al. (2025) investigated more than 300 experimental conditions involving microfluidically manufactured liposomes based on clinically inspired compositions [59]. Their study demonstrated the application of predictive models and a computational simulation tool to facilitate formulation-space exploration and guide experimental optimization.

Further progress has been achieved through the integration of ML with process analytical technologies (PAT). Lee et al. (2026) developed an in-line PAT-assisted ML framework incorporating physicochemical membrane characteristics to predict liposome particle size [60]. The model achieved a root mean square error of 7.18 nm, with an error of 7.53 nm during external validation under previously unseen experimental conditions. These findings illustrate the potential of combining real-time process measurements with predictive modeling to support liposome manufacturing and quality control.

Despite these advances, AI-assisted formulation development remains dependent on the availability, quality, and representativeness of experimental datasets. Predictive performance may decrease when models are applied to lipid compositions, processing conditions, or therapeutic cargos outside their validated domains. Furthermore, accurate prediction of physicochemical properties does not necessarily establish biological performance or long-term formulation stability. Therefore, ML should be considered complementary to DoE, mechanistic understanding, and experimental validation rather than a replacement for these established approaches. Their integration within a quality-by-design framework may support more efficient formulation screening, process optimization, and the development of reproducible liposomal pharmaceutical products.

4.3. Vesicle Size and Polydispersity Index

Having established the relationships between manufacturing process parameters and liposomal quality attributes, the following subsections discuss the principal physicochemical characteristics of liposomal formulations and the analytical techniques used for their evaluation. Among all liposomal attributes, vesicle size is one of the most influential factors because it affects circulation behavior, tissue distribution, cellular uptake, drug release, sterilization feasibility, and long-term physical stability. The size of nanoparticles also determines how a formulation interacts with filtration membranes, biological barriers, and the mononuclear phagocyte system. Accordingly, vesicle size is routinely measured during formulation optimization and batch release testing, most commonly by dynamic light scattering (DLS), which remains the standard method for the rapid size analysis of liposomes in suspension [51,61].

DLS measures the fluctuations in scattered light intensity produced by the Brownian motion of suspended particles. These temporal fluctuations are mathematically related to the translational diffusion coefficient, from which the hydrodynamic diameter is calculated through the Stokes–Einstein relationship. Importantly, the obtained value is the hydrodynamic diameter, not the dry geometric diameter, meaning that the measurement reflects not only the vesicle itself but also the solvent layer and any associated interfacial structures surrounding it. Because DLS is based on the scattered light intensity, larger particles or aggregates contribute disproportionately to the signal. As a result, DLS is highly sensitive to even a small fraction of oversized particles, which makes it extremely useful for detecting aggregation; however, the data must be interpreted with caution, especially in polydisperse samples [51,62].

The polydispersity index provides complementary information by describing the breadth of the size distribution derived from the DLS correlation function. In liposome research, lower PDI values generally indicate a narrower and more homogeneous vesicle population, whereas higher values suggest heterogeneity, coexistence of multiple subpopulations, or incipient aggregation. Although the exact acceptance threshold depends on the application and measurement protocol, PDI values below approximately 0.2 are commonly interpreted as reflecting a relatively narrow distribution, whereas values above about 0.3 usually indicate broader heterogeneity that may compromise reproducibility and predictability [51,56,63]. However, these thresholds should not be treated as absolute. Sample dilution, refractive index assumptions, viscosity input, dust contamination, and the presence of non-spherical structures can all affect the DLS output. Therefore, DLS is best used as a high-throughput and highly informative screening method, but not as the sole basis for structural conclusions. Nanoparticle Tracking Analysis (NTA) serves as a single-particle technique that complements DLS in the characterization of liposome size. In NTA, a laser is used to illuminate the sample, and a camera captures the light scattered by individual particles as they undergo Brownian motion [64]. By tracking the movement of each particle, the diffusion coefficient is determined, and the hydrodynamic diameter is subsequently calculated using the Stokes–Einstein equation. In contrast to DLS, NTA offers a number-based size distribution along with particle concentration, which enhances the resolution of polydisperse samples and facilitates the detection of aggregates [64]. Tunable resistive pulse sensing (TRPS) is a single-particle technique employed in conjunction with DLS for the characterization of liposomes [65]. In TRPS, particles suspended in an electrolyte are propelled through a nanopore with adjustable size. Each particle's passage through the pore results in a temporary decrease in ionic current, with the signal's magnitude being directly proportional to the particle's volume [65]. By calibrating with standard particles of known dimensions, TRPS yields number-based size distributions and particle concentrations, and it is capable of measuring the surface charge of individual

particles. Consequently, it serves as a valuable complementary method for evaluating liposome heterogeneity and stability [65].

4.4. Zeta Potential and Interfacial Stability

Zeta potential is widely used to assess the effective surface electrostatic behavior of liposomes in dispersion and remains one of the most informative physicochemical parameters for predicting colloidal stability [66]. More precisely, it represents the electrokinetic potential at the slipping plane, which is the conceptual boundary separating the liquid layer that moves with the particle from the surrounding bulk medium. Because this potential reflects the net result of the surface charge, ion adsorption, and structure of the electrical double layer, it provides indirect but valuable insights into the interparticle interactions in colloidal systems [67]. Consequently, the zeta potential is often interpreted as an indicator of the balance between repulsive and attractive forces and, thus, the likelihood of aggregation, fusion, or sedimentation during storage [66,67].

Measurement is typically performed using electrophoretic light scattering (ELS), where an applied electric field induces particle motion, and the resulting electrophoretic mobility is determined optically, commonly via laser Doppler velocimetry or phase analysis light scattering [68,69]. This experimentally measured mobility is then converted into zeta potential using theoretical models, such as the Smoluchowski or Henry equations, depending on the particle size and medium properties [69]. In liposomal systems, zeta potential is highly sensitive to formulation and environmental variables, including lipid composition (e.g., presence of charged phospholipids), pH, ionic strength, buffer composition, adsorption of proteins or other biomolecules, and the incorporation of surface modifiers such as PEGylated lipids [70]. Consequently, zeta potential values are not intrinsic constants but context-dependent measurements [71]. A formulation that appears strongly charged in low-ionic-strength media may exhibit significantly reduced apparent zeta potential in physiologically relevant buffers or protein-containing environments owing to charge screening and surface adsorption phenomena [70].

In practical terms, liposomes exhibiting a high absolute zeta potential are often considered less prone to aggregation because electrostatic repulsion counteracts attractive van der Waals forces [72]. A threshold value of approximately ±30 mV is frequently cited as indicative of good electrostatic stabilization; however, this guideline should be interpreted with caution and not as a universal criterion [6]. Many pharmaceutical liposomes achieve excellent physical stability at substantially lower absolute zeta potential values through steric stabilization mechanisms, particularly when PEGylated lipids or other surface-active polymers are present [73]. This consideration is especially important for so-called “stealth” liposomes, which are intentionally designed to possess near-neutral surface charge in order to minimize opsonization and prolong systemic circulation [70,73]. Conversely, highly cationic liposomes may demonstrate strong colloidal stability in vitro while presenting significant biological liabilities, including increased protein adsorption, rapid clearance by the mononuclear phagocyte system, and elevated cytotoxicity [74]. Therefore, zeta potential should be interpreted as a formulation-dependent indicator of behavior rather than as a definitive pass–fail parameter for stability or performance.

4.5. Morphology and Structural Visualization

Although DLS and zeta potential provide population-level information, microscopy is needed to directly visualize liposome architecture. Morphological characterization is particularly important when the goal is to distinguish unilamellar from multilamellar vesicles, confirm spherical versus irregular geometry, detect aggregation, or evaluate cargo-induced structural changes. In this context, cryogenic transmission electron microscopy

(cryo-TEM) has become the gold standard for lipid-based nanomedicine characterization [75].

The strength of cryo-TEM lies in its ability to preserve vesicles in a near-native hydrated state through rapid vitrification. This avoids many of the dehydration and staining artefacts associated with conventional TEM, such as collapse, flattening, or the "cup-shaped" appearance often seen with negative staining. By locking the lipids in a glassy ice layer, cryo-TEM reveals vesicle shape, internal structure, and sample heterogeneity at high resolution. This is especially critical for complex lipid-based systems, including payload-rich vesicles or mRNA lipid nanoparticles, where direct structural confirmation is essential to correlate with biological performance [75]. Despite these advantages, the accessibility of cryo-TEM remains limited by the high cost of specialized instrumentation, technically demanding cryogenic sample preparation, and the need for trained personnel [75]. Conventional negative-staining TEM offers a more accessible and relatively rapid alternative for morphological assessment, although sample preparation may introduce structural artefacts [76]. Therefore, cryo-TEM is particularly valuable when detailed information on vesicle morphology and lamellarity is required, while the choice of imaging technique should also consider instrument availability and analytical requirements.

Atomic force microscopy (AFM) offers complementary insights, particularly in the analysis of surface topography and nanomechanical properties. In contrast to cryo-TEM, AFM is especially effective for quantifying the roughness, stiffness, and deformation behavior of membranes. Consequently, it serves as an invaluable tool for examining elastic or deformable vesicular systems designed for topical delivery. However, it is important to note that this method is limited to surface analysis and is affected by sample immobilization [75].

Beyond direct imaging, complementary spectroscopic approaches can provide functional information on membrane organization and lipid–membrane interactions. Laurdan fluorescence can be used to assess changes in lipid bilayer fluidity, whereas Förster resonance energy transfer (FRET) can detect lipid mixing and membrane-fusion-associated structural changes. For example, incorporation of α-linolenic acid into phospholipid liposomes produced a concentration-dependent increase in membrane fluidity and promoted fusion with model and cellular membranes, demonstrating how alterations in lipid composition can directly influence liposomal membrane behavior and cellular interactions [5].

4.6. Purification of Liposomal Formulations

Following liposome formation, purification is often required to remove unencapsulated drug, residual organic solvent, free proteins or nucleic acids, and other low-molecular-weight components from the external aqueous phase. This step is particularly important before determining encapsulation efficiency, since inadequate separation of free and liposome-associated cargo may result in overestimation of the amount effectively incorporated within the formulation. An ideal purification method should therefore efficiently separate unencapsulated material while minimizing vesicle disruption, cargo leakage, dilution, and lipid loss.

Several techniques are commonly employed depending on the physicochemical properties of the liposomes and their cargo. Dialysis provides a simple size-based approach in which small free molecules diffuse across a semipermeable membrane while liposomes are retained, although the process can be relatively slow and may require large volumes of exchange medium. Size-exclusion chromatography separates liposomes from smaller solutes according to hydrodynamic size and can provide rapid and relatively gentle purification, but sample dilution and limited processing capacity may restrict its use at larger scales. Ultracentrifugation and centrifugal ultrafiltration are also widely used at the laboratory scale; however, high centrifugal forces, membrane interactions, or

concentration effects may alter vesicle properties or reduce product recovery. In contrast, ultrafiltration and tangential-flow filtration (TFF) are increasingly attractive for translational manufacturing because they permit simultaneous concentration, diafiltration, removal of free cargo, and buffer exchange while offering greater scalability and process control. Experimental studies have demonstrated effective removal of unencapsulated drug, proteins, and residual solvents using TFF while maintaining high liposome recovery and largely preserving vesicle characteristics [77,78]. The purification strategy must therefore be selected according to liposome size, cargo properties, formulation stability, and intended manufacturing scale, since the separation process itself can influence the apparent encapsulation efficiency and final product quality.

4.7. Encapsulation Efficiency and Drug Loading

Following appropriate separation of unencapsulated cargo, encapsulation efficiency (EE%) and drug loading (DL%) provide two of the most practical measures of formulation performance. Although the terms are sometimes used interchangeably, they describe different concepts. Encapsulation efficiency refers to the fraction of the initial drug dose that becomes associated with or entrapped within the liposomes, whereas drug loading describes the amount of incorporated drug relative to the carrier mass, usually total lipid. Both are important: a high EE% indicates efficient capture of the input drug, whereas an appropriate DL% determines whether the formulation is clinically and technologically meaningful [51,79].

The analytical challenge in EE determination is the accurate differentiation and quantification of encapsulated and non-encapsulated drug without significantly disrupting the liposomal vesicles. The optimal approach depends strongly on the physicochemical properties and localization of the cargo. For hydrophilic compounds, which are typically entrapped within the aqueous core, unencapsulated drug remains in the external aqueous phase and must be removed efficiently without inducing leakage from the vesicles. For lipophilic drugs, which preferentially partition into the lipid bilayer, the apparent EE may be high, although drug incorporation can alter membrane packing, fluidity, and phase behavior and thereby influence formulation stability. For amphiphilic or ionisable compounds, distribution between the aqueous core, lipid bilayer, vesicle interface, and external medium may be more complex, further complicating accurate determination of EE [79].

Determination of EE generally involves two complementary analytical steps: separation of free drug from liposome-associated drug, followed by quantitative measurement of the drug. The separation techniques described in the preceding section, including size-exclusion chromatography, dialysis, and centrifugal ultrafiltration, can also be employed for analytical purposes. However, their application in EE determination requires particular attention to potential analytical biases, including incomplete separation, drug adsorption, vesicle leakage, and sample losses. Comparative studies have demonstrated that substantially different EE values may be obtained for the same liposomal formulation depending on the separation procedure used [79,80]. Consequently, the selected method should be evaluated for separation efficiency, drug recovery, and preservation of liposomal integrity, and EE values should be interpreted in relation to the analytical procedure rather than assumed to be directly comparable across different studies.

Following separation, the free, encapsulated, or total drug must be quantified using a suitable analytical technique. High-performance liquid chromatography (HPLC) is among the most robust approaches because it provides high sensitivity and selectivity and can distinguish the drug from lipids, formulation excipients, and potential degradation products. In a conventional workflow, free drug may be measured in the separated external fraction, whereas total or encapsulated drug is quantified following disruption

of the liposomes using an appropriate solvent or detergent. More advanced chromatographic approaches can integrate separation and quantification within a single analytical procedure. For example, column-switching HPLC has been developed for simultaneous determination of free and encapsulated doxorubicin following direct injection of liposomal formulations, while nanoparticle-exclusion chromatography (nPEC) enables chromatographic separation and quantification of free drug from intact liposomal nanoparticles with minimal sample pretreatment [81,82].

UV–visible spectrophotometry provides a simpler and more accessible alternative when the encapsulated drug possesses adequate UV or visible absorbance and interference from lipids and other formulation components can be controlled. Drug concentration is typically determined from a validated calibration curve after separation of free drug or disruption of purified liposomes. UV-based methods have been successfully applied to determine drug loading and encapsulation in liposomal formulations, including ciprofloxacin-containing liposomes [83]. Nevertheless, UV–Vis generally offers lower analytical selectivity than chromatographic methods and may be affected by spectral overlap from excipients, degradation products, or light scattering from intact vesicles. Appropriate formulation blanks, calibration procedures, and liposome disruption where necessary are therefore important for reliable quantification. Accordingly, the reliability of an EE value depends not only on the method used to separate free and encapsulated drug but also on the analytical technique used for subsequent quantification [79].

4.8. In Vitro Release Behavior

In vitro release testing provides a functional link between structure and performance by indicating how rapidly and to what extent the encapsulated cargo leaves the liposomal system under defined conditions. In principle, release studies are intended to distinguish immediate leakage, surface-associated burst release, and sustained release from the vesicle interior or bilayer domain. However, in practice, release data are highly method-dependent, and the experimental setup can strongly influence the observed profile [51].

An effective release study must consider the biological context of the formulation. Factors such as media composition, sink conditions, pH, ionic strength, temperature, protein content, and the separation method employed during sampling can all influence release behavior. Liposomal formulations are often tested at physiological pH and temperature; however, additional conditions may be relevant depending on the target environment, such as acidic pH to mimic endosomal compartments or protein-containing media to model serum interactions. It is also important to distinguish true drug release from vesicle instability or methodological leakage caused by dialysis membranes, dilution effects, or repeated sampling. Accordingly, release experiments should be designed as mechanistic tests rather than purely descriptive curves [50,51].

Mathematical modelling can strengthen the interpretation of in vitro release profiles, provided that the selected model is appropriate for the formulation and experimental conditions. The models discussed here are in vitro release kinetic models that describe the cumulative amount or fraction of drug released from liposomes over time, rather than pharmacokinetic or pharmacodynamic (PK/PD) models. Commonly applied models include zero-order, first-order, Higuchi, Hixson–Crowell, Korsmeyer–Peppas, Gompertz, and Weibull models, which differ in their mathematical assumptions and applicability to particular release mechanisms. However, liposomes may exhibit biphasic or multiphasic release profiles, particularly when an initial burst is followed by slower release from the vesicle interior or lipid bilayer, or when release is triggered by external stimuli. Model fitting is therefore useful for quantitative comparison but should not be interpreted as definitive evidence of the underlying release mechanism or as a direct prediction of in vivo PK/PD behavior. For example, AlMajed et al. evaluated several mathematical models

for ultrasound-triggered drug release from targeted liposomes and demonstrated that model performance varied with ultrasound frequency and experimental conditions, with different models providing the best fits under different release conditions [84].

Overall, physicochemical characterization is not a set of disconnected measurements. Size and PDI indicate whether the vesicles are formed reproducibly; zeta potential provides insight into interfacial behavior and colloidal stability; cryo-TEM and AFM help confirm morphology and structural integrity; encapsulation efficiency and drug loading reveal how successfully the system carries the intended payload; and release testing connects these properties to the functional performance. When interpreted together rather than in isolation, these parameters provide an analytical basis for rational liposome development and meaningful comparisons across formulations [50,51,75], as summarized in Figure 4.

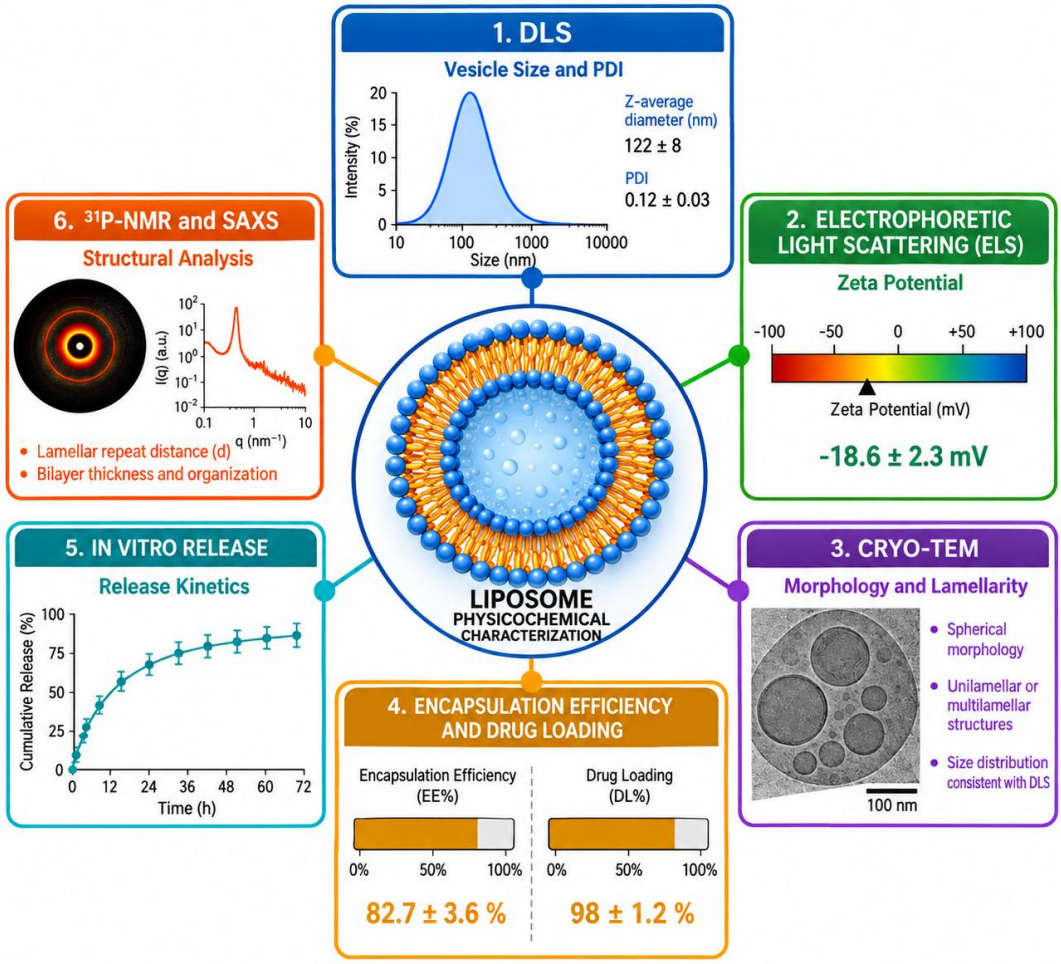


Figure 4. Physicochemical characterization workflow for liposomal systems. Schematic hub-and-spoke representation of key analytical techniques used to evaluate the critical quality attributes of liposomes. Dynamic light scattering (DLS) provides vesicle hydrodynamic diameter and polydispersity index (PDI), while electrophoretic light scattering (ELS) determines electrophoretic mobility, from which zeta potential is calculated. Cryogenic transmission electron microscopy (cryo-TEM) enables direct visualization of vesicle morphology and lamellarity. Encapsulation efficiency (EE%) quantifies the proportion of the initially added drug retained within or associated with liposomes after removal of unencapsulated drug, whereas drug loading (DL%) represents the amount of drug relative to the mass of the drug-loaded carrier. In vitro release studies assess drug release profiles under defined experimental conditions. Structural techniques, such as ^{31}P nuclear magnetic resonance (NMR) spectroscopy and small-angle X-ray scattering (SAXS), provide complementary insights into lipid bilayer organization, structural features, and lamellar arrangement. Together, these

analytical methods provide an integrated framework for assessing liposomal formulation quality, stability, and performance.

5. Lamellarity as a Structural and Functional Determinant

Lamellarity is a fundamental structural attribute that strongly influences the pharmaceutical performance, drug-loading behaviour, and release kinetics of liposomal delivery systems. It is most appropriately defined as the number of concentric lipid bilayers surrounding the internal aqueous compartment and exists on a structural continuum that includes unilamellar, oligolamellar, multilamellar, and multivesicular architectures. Liposomes are broadly classified according to both size and lamellarity into unilamellar vesicles (ULVs), oligolamellar vesicles (OLVs), multilamellar vesicles (MLVs), and multivesicular vesicles (MVVs/MVLs), as shown in Figure 1. Within the unilamellar class, small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) are the most commonly discussed pharmaceutical categories. Current reviews note that SUVs are widely used in parenteral products because their single-bilayer structure is generally better suited to systemic administration, while MLVs and MVVs provide expanded lipophilic membrane domains and may support depot-like behavior and altered release profiles [2].

The functional importance of lamellarity is especially evident in its control over encapsulation space and diffusional resistance. Unilamellar vesicles, by virtue of their single bilayer and comparatively high aqueous-core-to-lipid ratio, are generally favorable for predictable transport behavior and encapsulation of hydrophilic solutes within a defined internal compartment. In contrast, multilamellar vesicles exhibit a well-known "onion-like" arrangement of multiple concentric bilayers. Although this architecture reduces the effective aqueous trapped volume available per mole of lipid, it simultaneously increases the number of hydrophobic domains available for the incorporation of lipophilic drugs. Therefore, multilamellar systems may be advantageous when the formulation objective is prolonged retention or sustained local release rather than maximal aqueous loading efficiency [2,85].

From a mechanistic standpoint, lamellarity directly affects release kinetics through a diffusional barrier effect. In multilamellar vesicles, an encapsulated molecule must traverse sequential bilayers and intervening aqueous spaces before reaching the surrounding medium, which can substantially retard drug escape compared with that in a unilamellar system. This principle is one reason why multilamellar and multivesicular formulations are often considered attractive for sustained-release and depot applications. Multivesicular vesicles add a further level of internal complexity: rather than containing concentric shells, they are composed of multiple non-concentric internal aqueous compartments separated by lipid membranes, often described as a honeycomb-like internal organization. This multicompartmental structure can improve cargo retention and provide additional protection for sensitive payloads against premature degradation or redistribution [86,87].

Among the available analytical tools, ^{31}P nuclear magnetic resonance (NMR) remains one of the most chemically informative non-destructive methods for assessing lamellarity in phospholipid dispersions. Because phosphorus is an intrinsic constituent of phospholipid headgroups, ^{31}P-NMR can probe membrane organization directly. When a membrane-impermeable paramagnetic shift reagent, such as manganese (II) or certain lanthanide ions, is added to the external medium, the resonance arising from phospholipids exposed on the outer leaflet is selectively shifted or broadened, whereas phospholipids located in internal, bilayer-protected environments remain comparatively unperturbed. In principle, the relative intensities of the affected and unaffected signals can therefore provide an estimate of the fraction of externally accessible lipid and, by inference, the average lamellarity of the vesicle population. This general strategy has long been used as evidence

for unilamellarity in extruded vesicles, including the foundational work of Mayer and coworkers [15,85].

Simultaneously, ^{31}P NMR-based lamellarity analysis should not be presented as entirely assumption-free. Fröhlich et al. showed that the outcome can depend on experimental conditions, including buffer composition, ion concentration, reagent identity, and vesicle geometry, and concluded that ^{31}P-NMR may not always yield correct lamellarity values if these factors are not carefully controlled. For this reason, ^{31}P-NMR is highly valuable, but is best interpreted in conjunction with orthogonal structural methods rather than as a standalone absolute measure in all cases [85].

Small-angle X-ray scattering (SAXS) provides a complementary population-averaged structural method, particularly valuable for detecting lamellar order and repeat spacing. In multilamellar systems, the periodic stacking of bilayers gives rise to characteristic Bragg reflections, from which the lamellar repeat distance and related structural parameters can be extracted. By contrast, predominantly unilamellar systems produce broader scattering patterns with reduced long-range-order features. Modern SAXS analysis has been developed specifically for multilamellar vesicle systems and is sufficiently sensitive to resolve structural features even when only a limited number of diffraction orders are present, making it particularly useful for monitoring lamellar organization and batch consistency in manufacturing-oriented studies [88–90].

As discussed in the morphology section, cryo-TEM remains the most direct method for visual confirmation of lamellarity at the level of individual vesicles. Its main strength is that it can visually distinguish single-bilayer vesicles from oligolamellar, multilamellar, or more complex internal topologies that may be obscured in ensemble measurements, such as DLS. Recent work on lamellarity-controlled liposomes continues to use cryo-EM/cryo-TEM precisely for this confirmatory purpose, often alongside quantitative accessibility or fluorescence-based assays, because it reveals whether the measured population is structurally homogeneous or contains mixed vesicle classes [85,91].

However, because electron microscopy samples only a limited number of particles, it is most powerful when paired with bulk quantitative measures. In this context, captured-volume analysis remains a practical functional indicator of lamellarity. Perkins et al. emphasized that liposome systems can be described by interrelated parameters, including captured volume, vesicle size distribution, average lamellae number, and shape, and detailed methods for quantifying captured volume as a meaningful descriptor of the internal aqueous space. Likewise, Mayer et al. correlated trapped volume with vesicle preparation history and ^{31}P-NMR evidence of unilamellarity in extruded systems. Accordingly, when the experimentally measured internal aqueous volume falls substantially below the theoretical value expected for a unilamellar vesicle of comparable size, the result can serve as a practical indication that a significant fraction of the lipid is partitioned into internal lamellae rather than contributing to a single central aqueous core [15,90,92].

6. Quality Control and Liposomal Integrity

The clinical value of a liposomal formulation depends not only on its initial physicochemical profile but also on its ability to preserve that profile throughout storage, transport, sterilization, and administration. Accordingly, quality control in liposomal drug delivery is centred on maintaining vesicle integrity over time and under relevant processing conditions. From a practical perspective, liposomal instability can be divided into two broad categories: physical instability, which involves changes in vesicle organisation, such as aggregation, fusion, leakage, or size drift, and chemical instability, which involves the chemical degradation of membrane lipids, primarily through hydrolysis and oxidation, and, in some cases, degradation of the encapsulated payload [93]. These two forms of instability are closely linked, as chemical degradation of membrane components

may accelerate physical destabilisation, whereas physical changes in bilayer organization may expose lipids to hydrolytic or oxidative damage. The principal chemical degradation pathways of phospholipids, including hydrolysis and oxidation, and their consequences for bilayer integrity and cargo retention are illustrated in Figure 5. Therefore, quality control strategies for liposomes must be built around both structural preservation and chemical integrity [94].

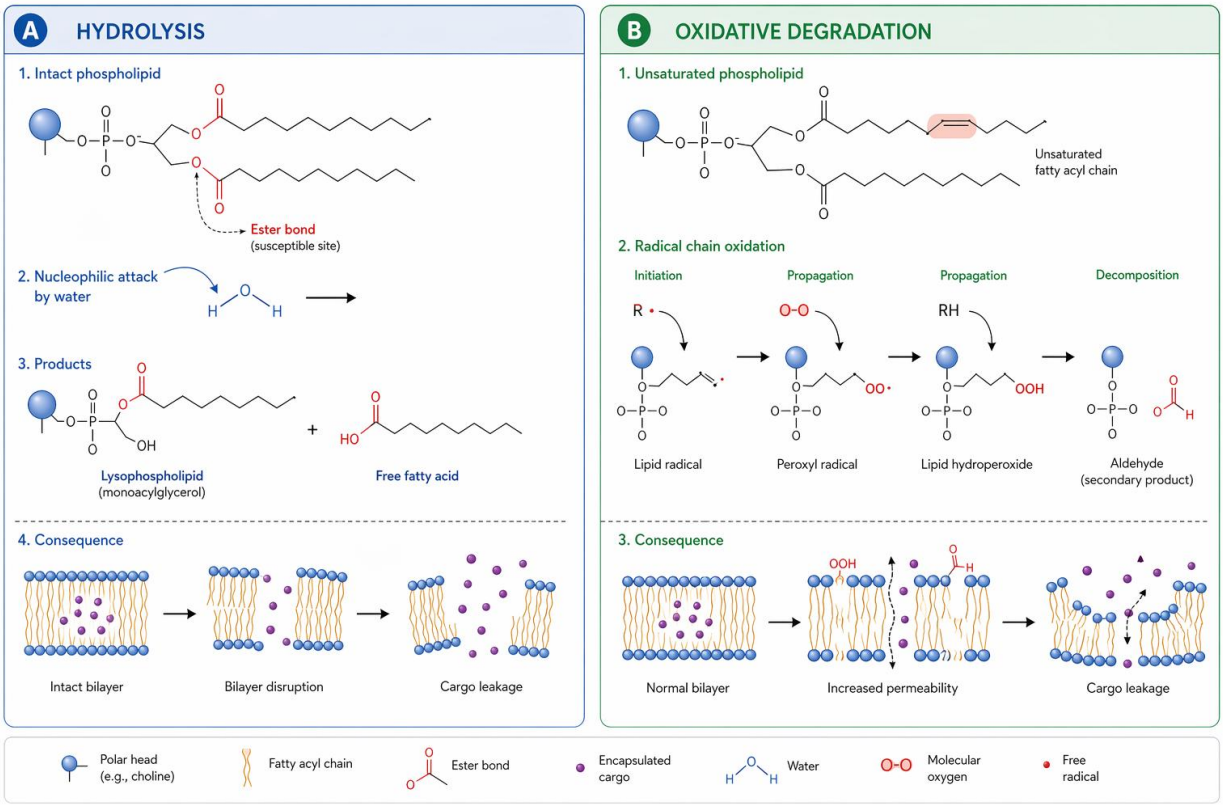


Figure 5. Chemical degradation pathways in liposomal systems. Schematic representation of the principal chemical degradation mechanisms affecting phospholipid bilayers. (**A**) Hydrolysis: Cleavage of phospholipid ester bonds through non-enzymatic reactions with water or enzyme-mediated processes, particularly phospholipase A2 (PLA2)-catalysed hydrolysis at the sn-2 position, resulting in the formation of lysophospholipids and free fatty acids. These products can destabilize the phospholipid bilayer, disrupt membrane integrity, and promote cargo leakage. (**B**) Oxidative degradation: Free radical-mediated oxidation of unsaturated phospholipid acyl chains in the presence of molecular oxygen, leading to the formation of lipid hydroperoxides and secondary degradation products, including aldehydes. These reactions alter bilayer organization, increase membrane permeability, and promote cargo leakage. Both degradation pathways can compromise the physicochemical stability of liposomal formulations during storage, handling, and exposure to biological environments.

6.1. Physical Instability: Aggregation, Fusion, and Leakage

Physical stability is primarily defined as the ability of liposomes to maintain their original size distribution, lamellarity, and colloidal dispersion state. Because liposomes are regarded as metastable assemblies with relatively high interfacial free energy, they are susceptible to aggregation, flocculation, fusion, and drug leakage. Liposome stability is influenced by surface charge and medium composition. Improved storage stability has been reported for some negatively charged formulations, but the effect depends on lipid composition and the surrounding medium, including ionic strength and salt composition

[95–98]. In one comparative study, aggregation and fusion were distinguished directly; Ca^{2+} was shown to promote coalescence or fusion, whereas La^{3+} was shown to promote aggregation [99]. In practice, physical instability is often detected first through an increase in mean particle size, broadening of the size distribution, or visible sedimentation or phase separation [95,98].

The classical colloid framework used for interpretation has been based on the balance between attractive van der Waals forces and repulsive forces, particularly electrostatic repulsion. In the studies retrieved here, this balance was shown to be strongly medium-dependent. In one study, the experimentally obtained zeta potentials were shown to agree with Gouy-Chapman behavior only under selected ionic conditions, and the experimentally observed stability was shown to deviate from theoretical expectations at low ionic strength in some cases [95,97]. Interpreting zeta potential is most effective when it is combined with direct stability tests, rather than being used as an independent indicator.

To improve physical stability, steric stabilization is often introduced, most commonly through PEGylation. The presence of a hydrophilic PEG layer can reduce nonspecific interactions with biological components and prolong liposome circulation. However, repeated administration of PEGylated liposomes may trigger the accelerated blood clearance (ABC) phenomenon, in which subsequent doses are cleared from the bloodstream more rapidly than the initial administration [100,101]. This phenomenon is commonly associated with an immune response in which the first administration induces anti-PEG immunoglobulin M (IgM) antibodies that recognize subsequently administered PEGylated liposomes, promoting complement activation and their uptake by the mononuclear phagocyte system, particularly in the liver and spleen [100,101]. Nevertheless, ABC is influenced by formulation characteristics and dosing conditions and is not exclusively determined by anti-PEG IgM levels. For example, a study involving maleimide-terminated PEGylated liposomes demonstrated accelerated clearance upon repeated administration despite lower anti-PEG IgM production compared with conventional PEGylated formulations [102]. Therefore, although PEGylation can improve colloidal stability and circulation persistence, its potential immunological consequences should be considered during repeated administration. Importantly, ABC represents an in vivo biological response rather than a direct manifestation of physical instability during storage.

In addition to PEGylation, physical stability is influenced by lipid phase behavior, cholesterol content, vesicle size, and storage conditions. In one storage study, the liposomes that mimicked platelet composition were found to be the most stable, and a net charge was associated with an extremely low likelihood of aggregation [95]. Across the literature retrieved here, the main practical message was that formulation variables and storage medium could shift a liposome from apparently stable to clearly unstable [95,97,98].

6.2. Chemical Stability: Hydrolysis and Oxidation

Chemical instability involves degradation of the lipid components of the bilayer, particularly through hydrolysis and oxidation. These changes may occur without detectable changes in vesicle size. For example, Zuidam and colleagues observed no changes in pH, vesicle size, or extent of oxidation following a standard autoclaving cycle, whereas phospholipid hydrolysis increased and encapsulated compounds leaked under certain experimental conditions [103]. This finding highlights the importance of assessing chemical integrity and cargo retention alongside physical stability.

In addition to non-enzymatic hydrolysis, phospholipids may undergo enzymatic degradation upon exposure to biological environments containing phospholipases. In particular, phospholipase A_2 (PLA_2) catalyses the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids, generating lysophospholipids and free fatty acids. The

accumulation of these products can alter lipid bilayer organization, increase membrane permeability, and promote premature release of encapsulated drugs [104]. However, susceptibility to enzymatic hydrolysis depends on lipid composition, membrane properties, and enzyme activity. Hansen et al. demonstrated that PLA_2-mediated liposomal degradation is influenced by phospholipid acyl-chain length, surface charge, temperature, and lipid-to-enzyme ratio [105]. Although enzymatic hydrolysis may compromise liposomal integrity in biological environments, this mechanism has also been investigated as a strategy for enzyme-triggered drug release at target sites [1].

Dry-state preservation, particularly lyophilisation, offers a strategy for improving storage stability, but its success depends on suitable protective excipients and optimized freezing, drying, and reconstitution conditions [106–110]. These formulation and processing considerations are discussed in Section 7.

6.3. Sterilization Challenges in Liposomal Formulations

Sterility is an essential requirement for parenteral liposomal formulations; however, terminal sterilisation can compromise liposome integrity. Zuidam and colleagues demonstrated that autoclaving was feasible for selected liposomal formulations, although phospholipid hydrolysis and leakage of encapsulated compounds depended on the experimental conditions and cargo properties [103]. Sterile filtration remains an important option when vesicle size and formulation properties permit it. Zourna and colleagues showed that filtration performance was strongly influenced by process conditions, liposome size, and size distribution [111]. Sterilisation should therefore be incorporated into formulation development rather than considered only as a final manufacturing step.

Overall, quality control of liposomal formulations should be understood as an integrated effort to preserve vesicle structure, lipid chemistry, cargo retention, and microbiological quality throughout manufacturing and storage. Physical stability testing should monitor aggregation, fusion, particle-size changes, and leakage, whereas chemical stability studies should assess phospholipid hydrolysis, oxidation, and cargo degradation. Dry-state stabilisation may further extend product stability, while sterilisation must be selected and optimized to avoid compromising liposome integrity [95,97–99,103,111–116].

7. Drying Techniques for Long-Term Stability

Long-term storage remains a major challenge for liposomal formulations because the aqueous environment can promote vesicle aggregation and fusion, drug leakage, phospholipid hydrolysis, and oxidative degradation [95,97–99]. Conversion of liposomal dispersions into a dry state can reduce molecular mobility and water-mediated degradation and is therefore widely investigated as a strategy to improve product stability and shelf life [112–114]. However, dehydration without appropriate protection can itself cause vesicle fusion and leakage, making the selection of protective excipients and drying conditions essential [112–114,117,118].

Freeze-drying (lyophilisation) and spray drying are two approaches to dry-state stabilisation of liposomal formulations. Freeze-drying removes water through ice sublimation followed by secondary drying, whereas spray drying involves atomisation of a liquid dispersion into a heated drying gas [115]. The following subsections discuss these techniques and their applications to liposomal systems, with selected examples from related lipid nanoparticles.

7.1. Freeze-Drying (Lyophilisation)

Lyophilisation consists of three principal stages: freezing, primary drying, and secondary drying. During freezing, the formation of ice excludes liposomes and dissolved solutes from the growing ice phase, concentrating them within the remaining unfrozen

fraction. These concentration effects may increase liposome–liposome interactions and induce fusion, aggregation, membrane disruption, or leakage. During primary drying, ice is removed by sublimation, whereas secondary drying further reduces residual moisture. Both freezing and dehydration can modify membrane phase behaviour, making optimization of the complete freeze-drying cycle essential for preservation of vesicle integrity [112–114,119].

Among the most widely investigated stabilizers are trehalose and sucrose. Early experimental work demonstrated that trehalose can preserve freeze-dried liposomes and inhibit dehydration-induced membrane fusion [112,113]. Subsequent studies demonstrated that carbohydrate-mediated stabilization involves both interactions between sugars and phospholipid headgroups and the formation of a highly viscous amorphous glass that limits molecular mobility in the dry state [117,118]. The importance of these mechanisms is reflected in studies showing that sucrose can reduce vesicle enlargement and drug leakage during lyophilisation of paclitaxel-loaded liposomes and can protect liposomes containing drugs located within the bilayer, although protection of hydrophilic cargo within the aqueous core may be more challenging [108,119].

The success of lyophilisation depends on the complete formulation rather than the choice of sugar alone. Freezing rate, lipid composition, cholesterol concentration, PEG content, cryoprotectant-to-lipid ratio, buffer composition, and residual moisture may all influence the final product. Van Winden and colleagues demonstrated that freezing rate significantly affected retention of an encapsulated marker following freeze-drying and rehydration [116] whereas a Quality-by-Design investigation of simvastatin-loaded liposomes identified interactions among lipid composition, cryoprotectant concentration, freezing conditions, vesicle size, drug retention, lipid phase-transition temperature, and residual moisture [120]. These findings emphasize the need to treat lyophilisation as a formulation- and process-specific operation rather than applying a universal cycle to different liposomal products.

The translational importance of this approach has become particularly apparent with the development of lipid-based vaccines and nucleic-acid therapeutics. The COVID-19 pandemic demonstrated the clinical potential of mRNA delivered by lipid nanoparticles but also highlighted the logistical difficulties associated with temperature-sensitive lipid–RNA formulations. More recent experimental studies have therefore investigated whether lyophilisation can reduce dependence on frozen storage.

Meulewaeter and colleagues demonstrated that optimized continuous freeze-drying of mRNA-LNPs preserved transfection activity for at least 12 weeks at 4, 22, and 37 °C, with formulation composition and buffer selection strongly influencing stability [121]. Ai and colleagues reported a lyophilized SARS-CoV-2 mRNA-LNP vaccine that retained physicochemical characteristics and biological activity during six months of storage at 25 °C and remained immunogenic in animal models and human evaluation [122]. A 2025 formulation-screening study, conducted primarily with polyA-loaded LNPs as surrogates and followed by testing with eGFP-mRNA, identified trehalose–PVP combinations as promising stabilizers. However, the eGFP-mRNA formulation showed reduced encapsulation efficiency following lyophilisation, highlighting the need to distinguish preservation during storage from damage incurred during the drying process [123].

Dry-state stabilization is also relevant to conventional liposomal vaccine adjuvants, not only mRNA-LNPs. AS01B is a liposome-based adjuvant used in Shingrix®, and experimental thin-film freeze-drying of AS01B-containing formulations using sucrose maintained the particle-size distribution of the liposomal adjuvant after reconstitution [124]. This distinction is important because it demonstrates that the principles of dry-state stabilization apply both to classical liposomal systems and to newer non-bilayer lipid nanoparticles.

7.2. Spray Drying

Spray drying provides an alternative approach in which a liquid liposomal dispersion is atomised into a heated drying gas, resulting in rapid solvent evaporation and formation of dry particles [115]. Unlike conventional batch freeze-drying, spray drying is a continuous process and can be readily scaled by increasing feed throughput or equipment capacity. It can also be used to engineer particle size, morphology, density, and aerodynamic properties, making it particularly attractive for pulmonary and mucosal formulations.

However, the atomisation and drying processes expose liposomes to shear, air–liquid interfaces, dehydration, and thermal stress, all of which may alter bilayer structure or promote leakage and aggregation. Appropriate excipient selection is therefore essential. Trehalose has been extensively investigated because its glass-forming properties can protect the liposomal membrane during rapid dehydration.

A representative experimental example is the cationic liposomal adjuvant CAF01. Ingvarsson and colleagues compared several excipients during spray drying and found that trehalose and lactose provided better preservation of liposome size than mannitol; importantly, optimized spray drying with trehalose also preserved the in vivo adjuvant activity of CAF01 [125]. More recent work with CAF04 liposomes showed that mixtures of trehalose and dextran could protect cationic liposomes during spray drying while allowing a relatively high liposome content in the dried powder [126].

The translational potential of this approach has also been demonstrated in vaccine delivery. In 2024, Rodrigues and colleagues produced DPPC/DC-Chol liposomes containing pneumococcal surface protein antigens by microfluidic manufacturing followed by spray drying with trehalose. The resulting nanocomposite microparticles preserved protein activity and generated broad protective immune responses following pulmonary immunization in mice [127]. This illustrates how two advanced manufacturing approaches discussed in this review—controlled microfluidic liposome formation and dry-state processing—can be integrated into a single formulation workflow.

8. Translational Considerations

Lyophilization and spray drying address the same fundamental challenge of converting physically and chemically vulnerable aqueous lipid dispersions into more stable solid products, but their pharmaceutical characteristics differ. Lyophilization generally subjects the formulation to limited thermal stress and is well established for sensitive biological products, but conventional freeze-drying is relatively slow and requires careful optimization of freezing and drying conditions. Spray drying is considerably faster and readily compatible with continuous manufacturing, but atomization, thermal exposure, and air–liquid interfaces impose additional stresses on lipid bilayers and biological cargo [108,112,121–128].

Successful drying should therefore not be judged solely by the physical appearance of the lyophilized cake or powder. Following reconstitution, CQAs should be compared with those of the original dispersion, including particle size, PDI, zeta potential, morphology, encapsulation efficiency, cargo integrity, and biological activity. For lyophilized systems, residual moisture, glass-transition behavior, and solid-state characteristics should also be assessed because these parameters influence molecular mobility and long-term storage stability [108,115,121–123,128,129].

Taken together, these studies demonstrate that dry-state processing should be integrated into formulation development rather than regarded simply as a final manufacturing operation. When formulation composition, protective excipients, and process parameters are appropriately optimized, lyophilization and spray drying can preserve liposomal structure and biological functionality, extend shelf life, simplify transportation, and

potentially reduce dependence on cold-chain infrastructure. The development of dried liposomal adjuvants and thermostable mRNA-LNP vaccines illustrates the direct translational relevance of these technologies to contemporary lipid-based medicines [112,121–127].

9. Ligand Targeting and Clinical Translation of Liposomal Systems

9.1. Ligand-Targeted Liposomes: Formulation Design, Characterization, and Translational Challenges

Ligand-targeted liposomes have been developed to improve the selective interaction of liposomal drug delivery systems with cells expressing specific surface receptors. Unlike conventional non-targeted liposomes, whose biodistribution is largely governed by vesicle composition, size, surface characteristics, and interactions with biological environments, ligand-functionalized liposomes incorporate recognition molecules designed to promote receptor-specific binding and, where applicable, cellular internalization. Common targeting ligands include antibodies and antibody fragments, peptides, aptamers, carbohydrates, and small molecules such as folic acid. Early studies by Kirpotin et al. demonstrated the preparation of sterically stabilized anti-HER2 immunoliposomes using antibody fragments conjugated to PEGylated lipids, establishing the importance of ligand attachment and surface presentation for receptor recognition [130]. Similarly, Gabizon et al. investigated folate-functionalized PEGylated liposomes and demonstrated enhanced binding to folate receptor-expressing cells, providing experimental evidence for receptor-mediated targeting using small-molecule ligands [131].

The incorporation of targeting ligands generally involves the conjugation of recognition molecules to functionalized phospholipids, often through polyethylene glycol (PEG) spacers extending from the liposomal bilayer. Covalent conjugation strategies, including thiol–maleimide coupling, enable the attachment of suitable antibodies, peptides, and other functional molecules to reactive lipid derivatives. Alternatively, ligand–PEG–lipid conjugates can be incorporated into preformed liposomes through post-insertion techniques. Zalipsky et al. demonstrated the preparation of PEG-grafted liposomes bearing peptide and oligosaccharide ligands, while Ishida et al. developed a post-insertion approach for incorporating antibody-conjugated PEG–lipids into preformed liposomal formulations with minimal drug leakage under the investigated conditions [132,133]. These strategies illustrate the importance of selecting conjugation methods that preserve ligand functionality while minimizing disruption of the liposomal membrane.

The physicochemical characteristics and biological performance of ligand-targeted liposomes depend on several formulation variables, including ligand density, molecular orientation, PEG spacer length, and accessibility of the targeting moiety. Kirpotin et al. demonstrated that the attachment position and density of anti-HER2 antibody fragments influenced immunoliposome binding and internalization [130]. Similarly, Gabizon et al. found that the presence and molecular weight of PEG chains influenced folate receptor binding, highlighting the potential for steric shielding when targeting ligands are insufficiently exposed beyond the polymer coating [131]. Consequently, ligand density and surface presentation must be optimized to achieve receptor recognition without compromising colloidal stability or introducing excessive interactions with biological components.

Experimental studies have also demonstrated that receptor-mediated targeting does not necessarily increase the total amount of liposomal material accumulating within a tumor. Mamot et al. reported that epidermal growth factor receptor (EGFR)-targeted immunoliposomes enhanced cellular internalization and antitumor activity despite comparable overall tumor accumulation to non-targeted formulations [134]. Similarly, Kirpotin et al. demonstrated that HER2-directed antibody targeting increased cellular internalization without significantly increasing total tumor localization in the investigated animal models

[135]. These findings emphasize an important distinction between tissue-level accumulation and receptor-mediated cellular delivery. Therefore, enhanced cellular uptake should not be interpreted as definitive evidence of improved tumor targeting or therapeutic efficacy without appropriate in vivo evaluation.

From a pharmaceutical development perspective, ligand functionalization introduces additional characterization and manufacturing requirements. Alongside conventional CQAs, including vesicle size, polydispersity, zeta potential, morphology, encapsulation efficiency, and drug release, targeted formulations require assessment of ligand conjugation efficiency, surface density, receptor-binding activity, and batch-to-batch consistency. Surface modification may also influence protein adsorption, circulation behavior, and immune recognition. For example, Guan et al. demonstrated that certain peptide-functionalized liposomes exhibited increased adsorption of natural IgM, resulting in altered immunocompatibility and clearance behavior [136]. Furthermore, the clinical investigation of HER2-targeted liposomal doxorubicin (MM-302) demonstrated the feasibility of translating antibody-functionalized liposomes into human studies, although subsequent development encountered limitations in clinical efficacy [137]. Overall, ligand-targeted liposomes offer opportunities to improve receptor-specific drug delivery, but their successful pharmaceutical translation requires integrated optimization of surface chemistry, physicochemical stability, manufacturing reproducibility, and biological performance.

9.2. Clinical Translation of Liposomal Drug Delivery Systems

The successful clinical translation of several liposomal formulations demonstrates the pharmaceutical importance of controlling vesicle composition, structural characteristics, drug loading, and manufacturing reproducibility. One of the earliest examples is PEGylated liposomal doxorubicin (Doxil), for which Gabizon et al. demonstrated prolonged circulation and accumulation in malignant exudates, providing important clinical evidence for the advantages of sterically stabilized liposomes [138]. Subsequent advances expanded the therapeutic applications of liposomal systems beyond conventional anticancer drug encapsulation. For example, the NAPOLI-1 trial demonstrated the clinical benefit of nanoliposomal irinotecan in combination with fluorouracil and folinic acid in previously treated metastatic pancreatic cancer [139]. Similarly, the CPX-351 formulation (Vyxeos), which co-encapsulates daunorubicin and cytarabine at a fixed synergistic molar ratio, demonstrated improved clinical outcomes compared with conventional chemotherapy in patients with secondary acute myeloid leukemia [140]. The clinical evaluation of inhaled liposomal amikacin further demonstrated the potential of liposomal formulations for localized pulmonary drug delivery [141].

Despite these advances, the transition from experimental liposomes to approved pharmaceutical products remains challenging. Several investigational formulations have reached clinical trials, including thermosensitive liposomes, cationic liposomal systems, and ligand-targeted formulations. For example, thermosensitive liposomal doxorubicin (ThermoDox) was evaluated in the Phase III HEAT trial in combination with radiofrequency ablation for hepatocellular carcinoma [142]. EndoTAG-1, a cationic liposomal paclitaxel formulation, was investigated clinically for its potential interaction with tumor vasculature [143]. Meanwhile, MM-302 and SGT-53 provide clinical examples of receptor-targeted liposomal delivery systems [137,144]. However, successful preclinical development and early clinical feasibility do not necessarily translate into regulatory approval or improved therapeutic outcomes. Table 3 summarizes representative approved liposomal products and selected investigational formulations evaluated in clinical trials, illustrating the diversity of formulation strategies and their translational outcomes.

Table 3. Representative approved liposomal products and investigational formulations evaluated in clinical trials.

Product	Active Ingredient	Main Application	Initial Approval	References
A. Regulatory-approved liposomal products				
Doxil/Caelyx	PEGylated liposomal doxorubicin	Cancer	FDA 1995	[145]
AmBisome	Liposomal amphotericin B	Systemic fungal infections	FDA 1997	[146]
Visudyne	Liposomal verteporfin	Photodynamic therapy	FDA 2000	[147]
Myocet	Non-PEGylated liposomal doxorubicin	Metastatic breast cancer	EU 2000	[148]
Exparel	Multivesicular liposomal bupivacaine	Postsurgical analgesia	FDA 2011	[149]
Onivyde	Liposomal irinotecan	Pancreatic cancer	FDA 2015	[139]
Vyxeos	Liposomal daunorubicin and cytarabine	Acute myeloid leukemia	FDA 2017	[140]
Arikayce	Liposomal amikacin	Refractory MAC pulmonary disease	FDA 2018	[141]
B. Selected investigational liposomal formulations evaluated in clinical trials				
ThermoDox	Thermosensitive liposomal doxorubicin	Hepatocellular carcinoma	Phase III HEAT trial; failed to meet its primary efficacy endpoint	[142]
EndoTAG-1	Cationic liposomal paclitaxel	Pancreatic cancer	Randomized Phase II trial published	[143]
MM-302	HER2-targeted liposomal doxorubicin	HER2-positive breast cancer	Phase I published; subsequent Phase II HERMIONE trial terminated after futility analysis	[137]
SGT-53	Transferrin receptor-targeted liposomal p53 gene-delivery complex	Advanced solid tumors	Phase I clinical study completed and published	[144]

10. Conclusion and Future Perspectives

Liposomes have evolved from simple phospholipid vesicles into versatile pharmaceutical delivery systems capable of incorporating small molecules, biologics, vaccines, and nucleic-acid therapeutics. Nevertheless, successful liposome development remains technically demanding because formulation performance depends on interactions among multiple variables. Lipid composition, preparation method, cargo-loading strategy, vesicle size, size distribution, surface properties, lamellarity, encapsulation efficiency, release behavior, and stability collectively influence the quality and functionality of the final product.

A major challenge remains the translation of promising laboratory formulations into reproducible and scalable pharmaceutical products. Conventional preparation methods remain valuable for formulation screening and mechanistic studies, although some require batch processing and post-formation manipulation that can complicate scale-up. Controlled-flow and microfluidic technologies offer opportunities to regulate vesicle formation more consistently, but their advantages depend on formulation-specific optimization and appropriate process controls. Scalability should therefore be considered from the early stages of development, with manufacturing parameters linked explicitly to the CQAs they influence. Advances in RNA-loaded lipid nanoparticle manufacturing may inform this process, provided that the structural and functional distinctions between these carriers and conventional liposomes are maintained.

Physicochemical characterization should establish meaningful relationships between formulation composition, processing conditions, structural organization, stability, and

functional performance. Measurements such as particle size, PDI, zeta potential, morphology, encapsulation efficiency, and drug release are most informative when interpreted together. Complementary analytical approaches are particularly important for complex attributes such as lamellarity, membrane organization, and cargo localization, for which no single technique provides a complete structural description.

Future research should also place greater emphasis on standardization and comparability. Differences in sample preparation, measurement conditions, purification procedures, release media, and data interpretation can produce substantially different results even for similar formulations. More consistent experimental and reporting practices would support reproducibility and meaningful comparison between studies. Establishing how CQAs relate to pharmaceutical performance and biological function should take precedence over reporting favorable numerical values without sufficient methodological or functional context.

Long-term stability represents another major translational priority. Aggregation, fusion, cargo leakage, phospholipid degradation, and sensitivity to sterilization can alter an otherwise optimized formulation during storage and manufacturing. Stability should therefore be incorporated into formulation design from the outset. Lyophilization and spray drying can improve storage stability and potentially reduce cold-chain dependence, but these benefits require formulation-specific evidence. Successful dry-state processing should be demonstrated through preservation of vesicle characteristics, cargo integrity, and relevant biological activity after reconstitution and throughout storage.

Ligand-targeted liposomes introduce additional opportunities and development requirements. Surface functionalization can improve receptor-specific binding and cellular internalization, but these effects do not necessarily translate into greater tissue accumulation or improved therapeutic outcomes. Ligand density, orientation, accessibility, and binding activity must therefore be evaluated alongside conventional quality attributes, manufacturing consistency, and interactions with biological environments. The experience of approved liposomal products and investigational formulations emphasizes that pharmaceutical translation requires evidence of clinical benefit together with reproducible product quality; promising preclinical performance alone is insufficient.

Looking forward, a systematic quality-by-design approach can support the identification and control of critical material attributes and process parameters that affect product quality. Scalable manufacturing, purification, complementary characterization, stability testing, and dry-state processing should be integrated throughout development. Future progress will depend on a clearer mechanistic understanding of how composition and processing influence structure, cargo behavior, stability, and biological performance. Connecting these elements from early formulation design through manufacturing and storage can support the development of more reproducible liposomal products and strengthen their progression toward clinical application.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/doi/s1.

Author Contributions: Conceptualization, A.A.A., M.J.A. and S.A.; literature review and data collection, A.A.A., M.J.A., H.A. (Hamoud Alotaibi), M.A.H., M.A.M., H.A. (Hajar Alghamdi) and R.A.; writing—original draft preparation, A.A.A., M.J.A., H.A. (Hamoud Alotaibi), M.A.H., M.A.M., H.A. (Hajar Alghamdi) and R.A.; writing—review and editing, A.A.A., M.J.A., H.A. (Hamoud Alotaibi), M.A.H., M.A.M., H.A. (Hajar Alghamdi), R.A. and S.A.; visualization and figure conceptualization, A.A.A.; supervision, S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

https://doi.org/10.3390/pharmaceutics18111412

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: During the preparation of this manuscript, the authors used ChatGPT (OpenAI; model: GPT-6 Astra) to improve grammar, consistency, and sentence flow, rather than to generate the manuscript's substantive content. The authors also used ChatGPT's image-generation functionality to create all figures and FigureLabs to generate the graphical abstract https://chat.figurelabs.ai/verify/FL-PUB-20261029-JZTD0E (accessed on 26 October 2026). The prompts used to generate the figures are provided in Supplementary Materials. The authors have reviewed and edited the generated output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Bangham, A.D.; Standish, M.M.; Watkins, J.C. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *J. Mol. Biol.* **1965**, *13*, 238–252, IN26–IN27. https://doi.org/10.1016/S0022-2836(65)80093-6.
2. Eugster, R.; Luciani, P. Liposomes: Bridging the gap from lab to pharmaceuticals. *Curr. Opin. Colloid Interface Sci.* **2025**, *75*, 101875. https://doi.org/10.1016/J.COCIS.2024.101875.
3. Fulton, M.D.; Najahi-Missaoui, W. Liposomes in Cancer Therapy: How Did We Start and Where Are We Now. *Int. J. Mol. Sci.* **2023**, *24*, 6615. https://doi.org/10.3390/IJMS24076615.
4. Israelachvili, J.N. *Intermolecular and Surface Forces*, 3rd ed.; Academic Press: Cambridge, MA, USA; 2011.
5. Aljasser, A.; Elbahr, R.; Bosquillon, C.; Stolnik, S. Fusion of liposomes incorporating α-linolenic acid with the cell plasma membrane is site-restricted. *Nanoscale* **2025**, *17*, 18377–18391. https://doi.org/10.1039/D5NR00560D.
6. Tenchov, R.; Bird, R.; Curtze, A.E.; Zhou, Q. Lipid Nanoparticles─From Liposomes to mRNA Vaccine Delivery, a Landscape of Research Diversity and Advancement. *ACS Nano* **2021**, *15*, 16982–17015. https://doi.org/10.1021/ACSNANO.1C04996.
7. Chelliah, R.; Rubab, M.; Vijayalakshmi, S.; Karuvelan, M.; Barathikannan, K.; Oh, D.H. Liposomes for drug delivery: Classification, therapeutic applications, and limitations. *Next Nanotechnol.* **2025**, *8*, 100209. https://doi.org/10.1016/J.NXNANO.2025.100209.
8. Bulbake, U.; Doppalapudi, S.; Kommineni, N.; Khan, W. Liposomal Formulations in Clinical Use: An Updated Review. *Pharmaceutics* **2017**, *9*, 12. https://doi.org/10.3390/PHARMACEUTICS9020012.
9. Della Camera, G.; Lipsa, D.; Mehn, D.; Italiani, P.; Boraschi, D.; Gioria, S. A Step-by-Step Approach to Improve Clinical Translation of Liposome-Based Nanomaterials, a Focus on Innate Immune and Inflammatory Responses. *Int. J. Mol. Sci.* **2021**, *22*, 820. https://doi.org/10.3390/IJMS22020820.
10. Liu, P.; Chen, G.; Zhang, J. A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. *Molecules* **2022**, *27*, 1372. https://doi.org/10.3390/MOLECULES27041372.
11. Sercombe, L.; Veerati, T.; Moheimani, F.; Wu, S.Y.; Sood, A.K. Advances and Challenges of Liposome Assisted Drug Delivery. *Front. Pharmacol.* **2015**, *6*, 163819. https://doi.org/10.3389/FPHAR.2015.00286.
12. Karn, P.R.; Jin, S.E.; Lee, B.J.; Sun, B.K.; Kim, M.S.; Sung, J.H.; Hwang, S.J. Preparation and evaluation of cyclosporin A-containing proliposomes: A comparison of the supercritical antisolvent process with the conventional film method. *Int. J. Nanomed.* **2014**, *9*, 5079–5091. https://doi.org/10.2147/IJN.S70340.
13. Basak, S.; Kanti Das, T. Liposome-Based Drug Delivery Systems: From Laboratory Research to Industrial Production—Instruments and Challenges. *ChemEngineering* **2025**, *9*, 2025. https://doi.org/10.3390/CHEMENGINEERING9030056.
14. Bnyan, R.; Cesarini, L.; Khan, I.; Roberts, M.; Ehtezazi, T. The effect of ethanol evaporation on the properties of inkjet produced liposomes. *DARU J. Pharm. Sci.* **2020**, *28*, 271–280. https://doi.org/10.1007/s40199-020-00340-1.
15. Mayer, L.D.; Hope, M.J.; Cullis, P.R. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta (BBA)-Biomembr.* **1986**, *858*, 161–168. https://doi.org/10.1016/0005-2736(86)90302-0.
16. Szoka, F.; Papahadjopoulos, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 4194–4198. https://doi.org/10.1073/PNAS.75.9.4194.

17. Mimms, L.T.; Zampighi, G.; Nozaki, Y.; Tanford, C.; Reynolds, J.A. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry* **1981**, *20*, 833–840. https://doi.org/10.1021/BI00507A028.
18. Massing, U.; Cicko, S.; Ziroli, V. Dual asymmetric centrifugation (DAC)—A new technique for liposome preparation. *J. Control. Release* **2008**, *125*, 16–24. https://doi.org/10.1016/J.JCONREL.2007.09.010.
19. Hirsch, M.; Ziroli, V.; Helm, M.; Massing, U. Preparation of small amounts of sterile siRNA-liposomes with high entrapping efficiency by dual asymmetric centrifugation (DAC). *J. Control. Release* **2009**, *135*, 80–88. https://doi.org/10.1016/J.JCONREL.2008.11.029.
20. Koehler, J.K.; Schnur, J.; Heerklotz, H.; Massing, U. Screening for Optimal Liposome Preparation Conditions by Using Dual Centrifugation and Time-Resolved Fluorescence Measurements. *Pharmaceutics* **2021**, *13*, 2046. https://doi.org/10.3390/PHARMACEUTICS13122046.
21. Gleue, L.; Graefen, B.; Voigt, M.; Schupp, J.; Schneider, D.; Fichter, M.; Kuske, M.; Mailaender, V.; Tuettenberg, A.; Helm, M. Dual Centrifugation-Based Screening for pH-Responsive Liposomes. *ChemMedChem* **2025**, *20*, e202400648. https://doi.org/10.1002/CMDC.202400648.
22. Gkionis, L.; Aojula, H.; Harris, L.K.; Tirella, A. Microfluidic-assisted fabrication of phosphatidylcholine-based liposomes for controlled drug delivery of chemotherapeutics. *Int. J. Pharm.* **2021**, *604*, 120711. https://doi.org/10.1016/J.IJPHARM.2021.120711.
23. Shepherd, S.J.; Issadore, D.; Mitchell, M.J. Microfluidic formulation of nanoparticles for biomedical applications. *Biomaterials* **2021**, *274*, 120826. https://doi.org/10.1016/J.BIOMATERIALS.2021.120826.
24. Wagner, A.; Vorauer-Uhl, K. Liposome Technology for Industrial Purposes. *J. Drug Deliv.* **2011**, *2011*, 591325. https://doi.org/10.1155/2011/591325.
25. Dhiman, N.; Sarvaiya, J.; Mohindroo, P. A drift on liposomes to proliposomes: Recent advances and promising approaches. *J. Liposome Res.* **2022**, *32*, 317–331. https://doi.org/10.1080/08982104.2021.2019762.
26. Choudhary, M.; Chaurawal, N.; Barkat, M.A.; Raza, K. Proliposome-Based Nanostrategies: Challenges and Development as Drug Delivery Systems. *AAPS PharmSciTech* **2022**, *23*, 293. https://doi.org/10.1208/S12249-022-02443-1.
27. Mitrović, D.; Zaklan, D.; Đanić, M.; Stanimirov, B.; Stankov, K.; Al-Salami, H.; Pavlović, N. The Pharmaceutical and Pharmacological Potential Applications of Bilosomes as Nanocarriers for Drug Delivery. *Molecules* **2025**, *30*, 1181. https://doi.org/10.3390/MOLECULES30051181.
28. Schoenmaker, L.; Witzigmann, D.; Kulkarni, J.A.; Verbeke, R.; Kersten, G.; Jiskoot, W.; Crommelin, D.J.A. MRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *Int. J. Pharm.* **2021**, *601*, 120586. https://doi.org/10.1016/J.IJPHARM.2021.120586.
29. Hou, X.; Zaks, T.; Langer, R.; Dong, Y. Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater.* **2021**, *6*, 1078–1094. https://doi.org/10.1038/s41578-021-00358-0.
30. Hassett, K.J.; Higgins, J.; Woods, A.; Levy, B.; Xia, Y.; Hsiao, C.J.; Acosta, E.; Almarsson, Ö.; Moore, M.J.; Brito, L.A. Impact of lipid nanoparticle size on mRNA vaccine immunogenicity. *J. Control. Release* **2021**, *335*, 237–246. https://doi.org/10.1016/J.JCONREL.2021.05.021.
31. Guevara, M.L.; Persano, F.; Persano, S. Advances in Lipid Nanoparticles for mRNA-Based Cancer Immunotherapy. *Front. Chem.* **2020**, *8*, 589959. https://doi.org/10.3389/FCHEM.2020.589959.
32. King, M.L. How manufacturing won or lost the COVID-19 vaccine race. *Vaccine* **2024**, *42*, 1004. https://doi.org/10.1016/J.VACCINE.2023.12.031.
33. Bethiana, T.; Aljabbari, A.; Li, Y.; Mitra, H.; Baghbanbashi, M.; Harris, G.S.; Dasaro, S.R.; Masoomi, F.; Vago, F.S.; Hartzler, S.L.; et al. Effects of different mixing techniques on mRNA lipid nanoparticle physicochemistry and biological performance. *Nat. Commun.* **2026**, *17*, 5890. https://doi.org/10.1038/s41467-026-72499-1.
34. Shepherd, S.J.; Han, X.; Mukalel, A.J.; El-Mayta, R.; Thatte, A.S.; Wu, J.; Padilla, M.S.; Alameh, M.G.; Srikumar, N.; Lee, D.; et al. Throughput-scalable manufacturing of SARS-CoV-2 mRNA lipid nanoparticle vaccines. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2303567120. https://doi.org/10.1073/PNAS.2303567120.
35. Jahn, A.; Vreeland, W.N.; Devoe, D.L.; Locascio, L.E.; Gaitan, M. Microfluidic Directed Formation of Liposomes of Controlled Size. *Langmuir* **2007**, *23*, 6289–6293. https://doi.org/10.1021/LA070051A.
36. Jahn, A.; Vreeland, W.N.; Gaitan, M.; Locascio, L.E. Controlled Vesicle Self-Assembly in Microfluidic Channels with Hydrodynamic Focusing. *J. Am. Chem. Soc.* **2004**, *126*, 2674–2675. https://doi.org/10.1021/JA0318030.
37. Kastner, E.; Kaur, R.; Lowry, D.; Moghaddam, B.; Wilkinson, A.; Perrie, Y. High-throughput manufacturing of size-tuned liposomes by a new microfluidics method using enhanced statistical tools for characterization. *Int. J. Pharm.* **2014**, *477*, 361–368. https://doi.org/10.1016/J.IJPHARM.2014.10.030.

38. Kastner, E.; Verma, V.; Lowry, D.; Perrie, Y. Microfluidic-controlled manufacture of liposomes for the solubilisation of a poorly water soluble drug. *Int. J. Pharm.* **2015**, *485*, 122–130. https://doi.org/10.1016/J.IJPHARM.2015.02.063.
39. Forbes, N.; Hussain, M.T.; Briuglia, M.L.; Edwards, D.P.; Horst, J.T.; Szita, N.; Perrie, Y. Rapid and scale-independent microfluidic manufacture of liposomes entrapping protein incorporating in-line purification and at-line size monitoring. *Int. J. Pharm.* **2019**, *556*, 68–81. https://doi.org/10.1016/J.IJPHARM.2018.11.060.
40. Webb, C.; Forbes, N.; Roces, C.B.; Anderluzzi, G.; Lou, G.; Abraham, S.; Ingalls, L.; Marshall, K.; Leaver, T.J.; Watts, J.A.; et al. Using microfluidics for scalable manufacturing of nanomedicines from bench to GMP: A case study using protein-loaded liposomes. *Int. J. Pharm.* **2020**, *582*, 119266. https://doi.org/10.1016/J.IJPHARM.2020.119266.
41. Roces, C.B.; Port, E.C.; Daskalakis, N.N.; Watts, J.A.; Aylott, J.W.; Halbert, G.W.; Perrie, Y. Rapid scale-up and production of active-loaded PEGylated liposomes. *Int. J. Pharm.* **2020**, *586*, 119566. https://doi.org/10.1016/J.IJPHARM.2020.119566.
42. Lindsay, S.; Holm, R.; Shah, S.; Perrie, Y. Impact of solvent choice during microfluidic manufacture on the in vivo performance of liposomal doxorubicin. *Drug Deliv. Transl. Res.* **2026**, *16*, 3702–3713. https://doi.org/10.1007/S13346-026-02062-4.
43. Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S.W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: Classification, preparation, and applications. *Nanoscale Res. Lett.* **2013**, *8*, 102. https://doi.org/10.1186/1556-276X-8-102.
44. Bozzuto, G.; Molinari, A. Liposomes as nanomedical devices. *Int. J. Nanomed.* **2015**, *10*, 975–999. https://doi.org/10.2147/IJN.S68861.
45. Li, J.; Wang, X.; Zhang, T.; Wang, C.; Huang, Z.; Luo, X.; Deng, Y. A review on phospholipids and their main applications in drug delivery systems. *Asian J. Pharm. Sci.* **2015**, *10*, 81–98. https://doi.org/10.1016/J.AJPS.2014.09.004.
46. Sharma, A.; Sharma, U.S. Liposomes in drug delivery: Progress and limitations. *Int. J. Pharm.* **1997**, *154*, 123–140.
47. Amoabediny, G.; Haghiralsadat, F.; Naderinezhad, S.; Helder, M.N.; Akhoundi Kharanaghi, E.; Mohammadnejad Arough, J.; Zandieh-Doulabi, B. Overview of preparation methods of polymeric and lipid-based (niosome, solid lipid, liposome) nanoparticles: A comprehensive review. *Int. J. Polym. Mater. Polym. Biomater.* **2018**, *67*, 383–400. https://doi.org/10.1080/00914037.2017.1332623.
48. Allen, T.M.; Cullis, P.R. Liposomal drug delivery systems: From concept to clinical applications. *Adv. Drug Deliv. Rev.* **2013**, *65*, 36–48. https://doi.org/10.1016/J.ADDR.2012.09.037.
49. Pattni, B.S.; Chupin, V.V.; Torchilin, V.P. New Developments in Liposomal Drug Delivery. *Chem. Rev.* **2015**, *115*, 10938–10966. https://doi.org/10.1021/acs.chemrev.5b00046.
50. Atre, P.; Rizvi, S.A.A. A brief overview of quality by design approach for developing pharmaceutical liposomes as nano-sized parenteral drug delivery systems. *RSC Pharm.* **2024**, *1*, 675–688. https://doi.org/10.1039/D4PM00201F.
51. Agrawal, S.S.; Baliga, V.; Londhe, V.Y. Liposomal Formulations: A Recent Update. *Pharmaceutics* **2024**, *17*, 36. https://doi.org/10.3390/PHARMACEUTICS17010036.
52. International Council for Harmonisation (ICH). Guidance for Industry Q8(R2) Pharmaceutical Development [Online]. 2009. Available online: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q8r2-pharmaceutical-development (accessed on 26 October 2026).
53. U.S. Food and Drug Administration (FDA). Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation|FDA. 2018. Available online: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/liposome-drug-products-chemistry-manufacturing-and-controls-human-pharmacokinetics-and (accessed on 26 October 2026).
54. Lombardo, D.; Kiselev, M.A. Methods of Liposomes Preparation: Formation and Control Factors of Versatile Nanocarriers for Biomedical and Nanomedicine Application. *Pharmaceutics* **2022**, *14*, 543. https://doi.org/10.3390/PHARMACEUTICS14030543.
55. Meure, L.A.; Foster, N.R.; Dehghani, F. Conventional and Dense Gas Techniques for the Production of Liposomes: A Review. *AAPS PharmSciTech* **2008**, *9*, 798–809. https://doi.org/10.1208/S12249-008-9097-X.
56. Hunter, D.G.; Frisken, B.J. Effect of Extrusion Pressure and Lipid Properties on the Size and Polydispersity of Lipid Vesicles. *Biophys. J.* **1998**, *74*, 2996–3002. https://doi.org/10.1016/S0006-3495(98)78006-3.
57. Khute, S.; Jangde, R.K. Optimization of Nasal Liposome Formulation of Venlafaxine Hydrochloride using a Box-Behnken Experimental Design. *Curr. Ther. Res.* **2023**, *99*, 100714. https://doi.org/10.1016/J.CURTHERES.2023.100714.
58. Eugster, R.; Orsi, M.; Buttitta, G.; Serafini, N.; Tiboni, M.; Casettari, L.; Reymond, J.L.; Aleandri, S.; Luciani, P. Leveraging machine learning to streamline the development of liposomal drug delivery systems. *J. Control. Release* **2024**, *376*, 1025–1038. https://doi.org/10.1016/J.JCONREL.2024.10.065.

59. Buttitta, G.; Lavagna, L.; Bonacorsi, S.; Barbarito, C.; Moliterno, M.; Saito, G.; Oddone, I.; Verdone, G.; Raimondi, S.; Panella, M. Machine Learning-Guided microfluidic optimization of clinically inspired liposomes for nanomedicine applications. *Int. J. Pharm.* **2025**, *686*, 126362. https://doi.org/10.1016/J.IJPHARM.2025.126362.
60. Lee, J.; Morishita Watanabe, N.; Yoshimoto, N.; Eom, S.; Kwak, M.K.; Jung, H.S.; Umakoshi, H. Liposome Particle Size Prediction by In-Line Process Analytical Technology (PAT)-Integrated Machine Learning. *Small Methods* **2026**, *10*, e70663. https://doi.org/10.1002/SMTD.70663.
61. Gómez-Hens, A.; Fernández-Romero, J.M. Analytical methods for the control of liposomal delivery systems. *TrAC-Trends Anal. Chem.* **2006**, *25*, 167–178. https://doi.org/10.1016/j.trac.2005.07.006.
62. Rodriguez-Loya, J.; Lerma, M.; Gardea-Torresdey, J.L. Dynamic Light Scattering and Its Application to Control Nanoparticle Aggregation in Colloidal Systems: A Review. *Micromachines* **2023**, *15*, 24. https://doi.org/10.3390/MI15010024.
63. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M.R. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **2018**, *10*, 57. https://doi.org/10.3390/PHARMACEUTICS10020057.
64. Filipe, V.; Hawe, A.; Jiskoot, W. Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. *Pharm. Res.* **2010**, *27*, 796–810. https://doi.org/10.1007/S11095-010-0073-2.
65. Willmott, G.R. Tunable Resistive Pulse Sensing: Better Size and Charge Measurements for Submicrometer Colloids. *Anal. Chem.* **2018**, *90*, 2987–2995. https://doi.org/10.1021/ACS.ANALCHEM.7B05106.
66. Németh, Z.; Csóka, I.; Semnani Jazani, R.; Sipos, B.; Haspel, H.; Kozma, G.; Kónya, Z.; Dobó, D.G. Quality by Design-Driven Zeta Potential Optimisation Study of Liposomes with Charge Imparting Membrane Additives. *Pharmaceutics* **2022**, *14*, 1798. https://doi.org/10.3390/PHARMACEUTICS14091798.
67. Lombardo, D.; Calandra, P.; Caccamo, M.T.; Magazù, S.; Kiselev, M.A. Colloidal stability of liposomes. *AIMS Mater. Sci.* **2019**, *6*, 200–213. https://doi.org/10.3934/MATERSCI.2019.2.200.
68. Gordillo-Galeano, A.; Mora-Huertas, C.E. Hydrodynamic diameter and zeta potential of nanostructured lipid carriers: Emphasizing some parameters for correct measurements. *Colloids Surf. A Physicochem. Eng. Asp.* **2021**, *620*, 126610. https://doi.org/10.1016/J.COLSURFA.2021.126610.
69. Bhattacharjee, S. DLS and zeta potential—What they are and what they are not? *J. Control. Release* **2016**, *235*, 337–351. https://doi.org/10.1016/J.JCONREL.2016.06.017.
70. Chen, Q.; Huang, Y.; Wu, C.; Pan, X.; Yu, C.; Wang, J.; Wang, W.; Huang, Z. Charge Effects: Influence of Surface Charge on Protein Corona Adsorption Behavior on Liposomal Formulations. *Pharmaceutics* **2026**, *18*, 76. https://doi.org/10.3390/pharmaceutics18010076.
71. Jarzynska, K.; Gajewicz-Skretna, A.; Ciura, K.; Puzyn, T. Predicting zeta potential of liposomes from their structure: A nano-QSPR model for DOPE, DC-Chol, DOTAP, and EPC formulations. *Comput Struct. Biotechnol. J.* **2024**, *25*, 3–8. https://doi.org/10.1016/J.CSBJ.2024.01.012.
72. Heurtault, B.; Saulnier, P.; Pech, B.; Proust, J.E.; Benoit, J.P. Physico-chemical stability of colloidal lipid particles. *Biomaterials* **2003**, *24*, 4283–4300. https://doi.org/10.1016/S0142-9612(03)00331-4.
73. Ni, Y.; Tang, S.; Wu, H.; Zhang, Y.; Tang, S.; Chen, L.; Lu, T.; Xu, D.; Hu, H. PEGylated liposomes of dimethylcurcumin: Preparation, in vitro antitumor effect and in vivo pharmacokinetics. *J. Dispers. Sci. Technol.* **2026**, 1–11. https://doi.org/10.1080/01932691.2026.2649020.
74. Inglut, C.T.; Sorrin, A.J.; Kuruppu, T.; Vig, S.; Cicalo, J.; Ahmad, H.; Huang, H.C. Immunological and Toxicological Considerations for the Design of Liposomes. *Nanomaterials* **2020**, *10*, 190. https://doi.org/10.3390/NANO10020190.
75. Takechi-Haraya, Y. Cryogenic Transmission Electron Microscopy for the Evaluation of Lipid-Based Nanomedicines: Principles, Applications, and Challenges. *Biol. Pharm. Bull.* **2025**, *48*, 1641–1651. https://doi.org/10.1248/BPB.B25-00158.
76. Baxa, U. Imaging of Liposomes by Transmission Electron Microscopy. *Methods Mol. Biol.* **2018**, *1682*, 73–88. https://doi.org/10.1007/978-1-4939-7352-1_8.
77. Dimov, N.; Kastner, E.; Hussain, M.; Perrie, Y.; Szita, N. Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. *Sci. Rep.* **2017**, *7*, 12045. https://doi.org/10.1038/s41598-017-11533-1.
78. Wagner, A.; Vorauer-Uhl, K.; Katinger, H. Liposomes produced in a pilot scale: Production, purification and efficiency aspects. *Eur. J. Pharm. Biopharm.* **2002**, *54*, 213–219. https://doi.org/10.1016/S0939-6411(02)00062-0.
79. Wang, J.P.; Huang, Z.R.; Zhang, C.; Ni, Y.R.; Li, B.T.; Wang, Y.; Wu, J.F. Methodological advances in liposomal encapsulation efficiency determination: Systematic review and analysis. *J. Drug Target.* **2025**, *33*, 1262–1271. https://doi.org/10.1080/1061186X.2025.2484773.

80. Ran, C.; Chen, D.; Xu, M.; Du, C.; Li, Q.; Jiang, Y. A study on characteristic of different sample pretreatment methods to evaluate the entrapment efficiency of liposomes. *J. Chromatogr. B* **2016**, *1028*, 56–62. https://doi.org/10.1016/J.JCHROMB.2016.06.008.
81. Ohnishi, N.; Yamamoto, E.; Tomida, H.; Hyodo, K.; Ishihara, H.; Kikuchi, H.; Tahara, K.; Takeuchi, H. Rapid determination of the encapsulation efficiency of a liposome formulation using column-switching HPLC. *Int. J. Pharm.* **2013**, *441*, 67–74. https://doi.org/10.1016/J.IJPHARM.2012.12.019.
82. Bian, J.; Girotti, J.; Fan, Y.; Levy, E.S.; Zang, N.; Sethuraman, V.; Kou, P.; Zhang, K.; Gruenhagen, J.; Lin, J. Fast and versatile analysis of liposome encapsulation efficiency by nanoParticle exclusion chromatography. *J. Chromatogr. A* **2022**, *1662*, 462688. https://doi.org/10.1016/J.CHROMA.2021.462688.
83. Gürsoy, A.; Senyücel, B. Characterization of ciprofloxacin liposomes: Derivative ultraviolet spectrophotometric determinations. *J. Microencapsul.* **1997**, *14*, 769–776. https://doi.org/10.3109/02652049709006827.
84. AlMajed, Z.; Salkho, N.M.; Sulieman, H.; Husseini, G.A. Modeling of the In Vitro Release Kinetics of Sonosensitive Targeted Liposomes. *Biomedicines* **2022**, *10*, 3139. https://doi.org/10.3390/BIOMEDICINES10123139.
85. Fröhlich, M.; Brecht, V.; Peschka-Süss, R. Parameters influencing the determination of liposome lamellarity by 31P-NMR. *Chem. Phys. Lipids* **2001**, *109*, 103–112. https://doi.org/10.1016/S0009-3084(00)00220-6.
86. Giuliano, C.B.; Cvjetan, N.; Ayache, J.; Walde, P. Multivesicular Vesicles: Preparation and Applications. *ChemSystemsChem* **2021**, *3*, e2000049. https://doi.org/10.1002/SYST.202000049.
87. Sreelaya, P.; Bhattacharya, S. A Mini-review Based on Multivesicular Liposomes: Composition, Design, Preparation, Characteristics, and Therapeutic Importance as DEPOFOAM® Technology. *Curr. Pharm. Biotechnol.* **2023**, *24*, 1479–1488. https://doi.org/10.2174/1389201024666230201090814.
88. Pabst, G.; Rappolt, M.; Amenitsch, H.; Laggner, P. Structural information from multilamellar liposomes at full hydration: Full q-range fitting with high quality x-ray data. *Phys. Rev. E* **2000**, *62*, 4000. https://doi.org/10.1103/PhysRevE.62.4000.
89. Heftberger, P.; Kollmitzer, B.; Heberle, F.A.; Pan, J.; Rappolt, M.; Amenitsch, H.; Kučerka, N.; Katsaras, J.; Pabst, G. Global small-angle X-ray scattering data analysis for multilamellar vesicles: The evolution of the scattering density profile model. *J. Appl. Crystallogr.* **2013**, *47*, 173–180. https://doi.org/10.1107/S1600576713029798.
90. Fan, Y.; Marioli, M.; Zhang, K. Analytical characterization of liposomes and other lipid nanoparticles for drug delivery. *J. Pharm. BioMed Anal.* **2021**, *192*, 113642. https://doi.org/10.1016/J.JPBA.2020.113642.
91. Koehler, J.K.; Gedda, L.; Wurster, L.; Schnur, J.; Edwards, K.; Heerklotz, H.; Massing, U. Tailoring the Lamellarity of Liposomes Prepared by Dual Centrifugation. *Pharmaceutics* **2023**, *15*, 706. https://doi.org/10.3390/PHARMACEUTICS15020706.
92. Perkins, W.R.; Minchey, S.R.; Ahl, P.L.; Janoff, A.S. The determination of liposome captured volume. *Chem. Phys. Lipids* **1993**, *64*, 197–217. https://doi.org/10.1016/0009-3084(93)90066-C.
93. Grit, M.; Crommelin, D.J.A. Chemical stability of liposomes: Implications for their physical stability. *Chem. Phys. Lipids* **1993**, *64*, 3–18. https://doi.org/10.1016/0009-3084(93)90053-6.
94. Zuidam, N.J.; Gouw, H.K.M.E.; Barenholz, Y.; Crommelin, D.J.A. Physical (in) stability of liposomes upon chemical hydrolysis: The role of lysophospholipids and fatty acids. *Biochim. Biophys. Acta (BBA)-Biomembr.* **1995**, *1240*, 101–110. https://doi.org/10.1016/0005-2736(95)00180-5.
95. Casals, E.; Galán, A.M.; Escolar, G.; Gallardo, M.; Estelrich, J. Physical stability of liposomes bearing hemostatic activity. *Chem. Phys. Lipids* **2003**, *125*, 139–146. https://doi.org/10.1016/S0009-3084(03)00086-0.
96. Sabín, J.; Prieto, G.; Ruso, J.M.; Hidalgo-Álvarez, R.; Sarmiento, F. Size and stability of liposomes: A possible role of hydration and osmotic forces. *Eur. Phys. J. E* **2006**, *20*, 401–408. https://doi.org/10.1140/epje/i2006-10029-9.
97. Carrión, F.J.; De La Maza, A.; Parra, J.L. The Influence of Ionic Strength and Lipid Bilayer Charge on the Stability of Liposomes. *J. Colloid Interface Sci.* **1994**, *164*, 78–87. https://doi.org/10.1006/JCIS.1994.1145.
98. Crommelin, D.J.A. Influence of Lipid Composition and Ionic Strength on the Physical Stability of Liposomes. *J. Pharm. Sci.* **1984**, *73*, 1559–1563. https://doi.org/10.1002/JPS.2600731118.
99. Sabín, J.; Prieto, G.; Messina, P.V.; Ruso, J.M.; Hidalgo-Alvarez, R.; Sarmiento, F. On the Effect of Ca^{2+} and La^{3+} on the Colloidal Stability of Liposomes. *Langmuir* **2005**, *21*, 10968–10975. https://doi.org/10.1021/LA051397T.
100. Ishida, T.; Harada, M.; Xin, Y.W.; Ichihara, M.; Irimura, K.; Kiwada, H. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: Effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. *J. Control. Release* **2005**, *105*, 305–317. https://doi.org/10.1016/J.JCONREL.2005.04.003.
101. Storm, G.; Laverman, P.; Dams, E.T.M.; Oussoren, C.; Corstens, F.H.M.; Oyen, W.J.G.; Boerman, O.C. Preclinical and Clinical Evidence for Efficient Opsonization of Poly(Ethyleneglycol)-Liposomes. *J. Liposome Res.* **2000**, *10*, 461–466. https://doi.org/10.3109/08982100009031111.

102. Ishima, Y.; Yamazaki, N.; Chuang, V.T.G.; Shimizu, T.; Ando, H.; Ishida, T. A Maleimide-Terminally Modified PEGylated Liposome Induced the Accelerated Blood Clearance Independent of the Production of Anti-PEG IgM Antibodies. *Biol. Pharm. Bull.* **2022**, *45*, 1518–1524. https://doi.org/10.1248/BPB.B22-00389.
103. Zuidam, N.J.; Lee, S.S.L.; Crommelin, D.J.A. Sterilization of Liposomes by Heat Treatment. *Pharm. Res. Off. J. Am. Assoc. Pharm. Sci.* **1993**, *10*, 1591–1596. https://doi.org/10.1023/a:1018916518515.
104. Davidsen, J.; Jørgensen, K.; Andresen, T.L.; Mouritsen, O.G. Secreted phospholipase A2 as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue. *Biochim. Biophys. Acta (BBA)-Biomembr.* **2003**, *1609*, 95–101. https://doi.org/10.1016/S0005-2736(02)00659-4.
105. Hansen, A.H.; Mouritsen, O.G.; Arouri, A. Enzymatic action of phospholipase A2 on liposomal drug delivery systems. *Int. J. Pharm.* **2015**, *491*, 49–57. https://doi.org/10.1016/J.IJPHARM.2015.06.005.
106. Ausborn, M.; Schreier, H.; Brezesinski, G.; Fabian, H.; Meyer, H.W.; Nuhn, P. The protective effect of free and membrane-bound cryoprotectants during freezing and freeze-drying of liposomes. *J. Control. Release* **1994**, *30*, 105–116. https://doi.org/10.1016/0168-3659(94)90257-7.
107. Izutsu, K.I.; Yomota, C.; Kawanishi, T. Stabilization of Liposomes in Frozen Solutions Through Control of Osmotic Flow and Internal Solution Freezing by Trehalose. *J. Pharm. Sci.* **2011**, *100*, 2935–2944. https://doi.org/10.1002/JPS.22518.
108. Guimarães, D.; Noro, J.; Silva, C.; Cavaco-Paulo, A.; Nogueira, E. Protective Effect of Saccharides on Freeze-Dried Liposomes Encapsulating Drugs. *Front. Bioeng. Biotechnol.* **2019**, *7*, 481516. https://doi.org/10.3389/fbioe.2019.00424.
109. Susa, F.; Bucca, G.; Limongi, T.; Cauda, V.; Pisano, R. Enhancing the preservation of liposomes: The role of cryoprotectants, lipid formulations and freezing approaches. *Cryobiology* **2021**, *98*, 46–56. https://doi.org/10.1016/J.CRYOBIOL.2020.12.009.
110. Liu, B.; Liu, Z.; Wang, P.; Huang, Z.; Wang, Q.; Zhang, Y.; He, H.; Yin, T.; Gou, J.; Tang, X. Potential of trehalose and sucrose-raffinose combination instead of sucrose applied to the cytarabine/daunorubicin co-loaded lyophilized liposome: Investigation on the protective effect and mechanism of various lyoprotectants. *J. Drug Deliv. Sci. Technol.* **2024**, *92*, 105360. https://doi.org/10.1016/J.JDDST.2024.105360.
111. Zourna, K.; Iwaniec, A.; Turner, S.; Jackson, N.B.; Welsh, J.H. Optimizing the Filtration of Liposomes Using Sterilizing-Grade Filters. *PDA J. Pharm. Sci. Technol.* **2021**, *75*, 128–140. https://doi.org/10.5731/PDAJPST.2020.011866.
112. Van Winden, E.C.A.; Zhang, W.; Crommelin, D.J.A. Effect of freezing rate on the stability of liposomes during freeze-drying and rehydration. *Pharm. Res.* **1997**, *14*, 1151–1160. https://doi.org/10.1023/A:1012142520912.
113. Kannan, V.; Balabathula, P.; Thoma, L.A.; Wood, G.C. Effect of sucrose as a lyoprotectant on the integrity of paclitaxel-loaded liposomes during lyophilization. *J. Liposome Res.* **2015**, *25*, 270–278. https://doi.org/10.3109/08982104.2014.992023.
114. Womersley, C.; Uster, P.S.; Rudolph, A.S.; Crowe, J.H. Inhibition of dehydration-induced fusion between liposomal membranes by carbohydrates as measured by fluorescence energy transfer. *Cryobiology* **1986**, *23*, 245–255. https://doi.org/10.1016/0011-2240(86)90050-7.
115. Crowe, L.M.; Reid, D.S.; Crowe, J.H. Is trehalose special for preserving dry biomaterials? *Biophys. J.* **1996**, *71*, 2087–2093. https://doi.org/10.1016/S0006-3495(96)79407-9.
116. Madden, T.D.; Bally, M.B.; Hope, M.J.; Cullis, P.R.; Schieren, H.P.; Janoff, A.S. Protection of large unilamellar vesicles by trehalose during dehydration: Retention of vesicle contents. *BBA-Biomembr.* **1985**, *817*, 67–74. https://doi.org/10.1016/0005-2736(85)90069-0.
117. Franzé, S.; Selmin, F.; Samaritani, E.; Minghetti, P.; Cilurzo, F. Lyophilization of Liposomal Formulations: Still Necessary, Still Challenging. *Pharmaceutics* **2018**, *10*, 139. https://doi.org/10.3390/PHARMACEUTICS10030139.
118. Yu, J.Y.; Chuesiang, P.; Shin, G.H.; Park, H.J. Post-Processing Techniques for the Improvement of Liposome Stability. *Pharmaceutics* **2021**, *13*, 1023. https://doi.org/10.3390/PHARMACEUTICS13071023.
119. Crowe, L.M.; Crowe, J.H.; Rudolph, A.; Womersley, C.; Appel, L. Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem Biophys.* **1985**, *242*, 240–247. https://doi.org/10.1016/0003-9861(85)90498-9.
120. Ingvarsson, P.T.; Yang, M.; Nielsen, H.; Rantanen, J.; Foged, C. Stabilization of liposomes during drying. *Expert Opin. Drug Deliv.* **2011**, *8*, 375–388. https://doi.org/10.1517/17425247.2011.553219.
121. Meulewaeter, S.; Nuytten, G.; Cheng, M.H.Y.; De Smedt, S.C.; Cullis, P.R.; De Beer, T.; Lentacker, I.; Verbeke, R. Continuous freeze-drying of messenger RNA lipid nanoparticles enables storage at higher temperatures. *J. Control. Release* **2023**, *357*, 149–160. https://doi.org/10.1016/j.jconrel.2023.03.039.
122. Ai, L.; Li, Y.; Zhou, L.; Yao, W.; Zhang, H.; Hu, Z.; Han, J.; Wang, W.; Wu, J.; Xu, P.; et al. Lyophilized mRNA-lipid nanoparticle vaccines with long-term stability and high antigenicity against SARS-CoV-2. *Cell Discov.* **2023**, *9*, 9. https://doi.org/10.1038/S41421-022-00517-9.

123. Ruppl, A.; Kiesewetter, D.; Köll-Weber, M.; Lemazurier, T.; Süss, R.; Allmendinger, A. Formulation screening of lyophilized mRNA-lipid nanoparticles. *Int. J. Pharm.* **2025**, *671*, 125272. https://doi.org/10.1016/j.ijpharm.2025.125272.
124. AboulFotouh, K.; Xu, H.; Moon, C.; Williams, R.O.; Cui, Z. Development of (Inhalable) Dry Powder Formulations of AS01B-Containing Vaccines Using Thin-Film Freeze-Drying. *Int. J. Pharm.* **2022**, *622*, 121825. https://doi.org/10.1016/j.ijpharm.2022.121825.
125. Ingvarsson, P.T.; Schmidt, S.T.; Christensen, D.; Larsen, N.B.; Hinrichs, W.L.J.; Andersen, P.; Rantanen, J.; Nielsen, H.M.; Yang, M.; Foged, C. Designing CAF-adjuvanted dry powder vaccines: Spray drying preserves the adjuvant activity of CAF01. *J. Control. Release* **2013**, *167*, 256–264. https://doi.org/10.1016/j.jconrel.2013.01.031.
126. Lutta, A.; Knopp, M.M.; Tollemeto, M.; Pedersen, G.K.; Schmidt, S.T.; Grohganz, H.; Hagner Nielsen, L. The interplay between trehalose and dextran as spray drying precursors for cationic liposomes. *Int. J. Pharm.* **2024**, *652*, 123798. https://doi.org/10.1016/j.ijpharm.2024.123798.
127. Rodrigues, T.C.; Figueiredo, D.B.; Gonçalves, V.M.; Kaneko, K.; Saleem, I.Y.; Miyaji, E.N. Liposome-based dry powder vaccine immunization targeting the lungs induces broad protection against pneumococcus. *J. Control. Release* **2024**, *368*, 184–198. https://doi.org/10.1016/j.jconrel.2024.02.028.
128. Porfire, A.; Muntean, D.M.; Rus, L.; Sylvester, B.; Tomuţă, I. A quality by design approach for the development of lyophilized liposomes with simvastatin. *Saudi Pharm. J.* **2017**, *25*, 981–992. https://doi.org/10.1016/j.jsps.2017.01.007.
129. Sun, W.Q.; Leopold, A.C.; Crowe, L.M.; Crowe, J.H. Stability of dry liposomes in sugar glasses. *Biophys. J.* **1996**, *70*, 1769–1776. https://doi.org/10.1016/S0006-3495(96)79740-0.
130. Kirpotin, D.; Park, J.W.; Hong, K.; Zalipsky, S.; Li, W.L.; Carter, P.; Benz, C.C.; Papahadjopoulos, D. Sterically Stabilized Anti-HER2 Immunoliposomes: Design and Targeting to Human Breast Cancer Cells in Vitro. *Biochemistry* **1997**, *36*, 66–75. https://doi.org/10.1021/BI962148U.
131. Gabizon, A.; Horowitz, A.T.; Goren, D.; Tzemach, D.; Mandelbaum-Shavit, F.; Qazen, M.M.; Zalipsky, S. Targeting Folate Receptor with Folate Linked to Extremities of Poly(ethylene glycol)-Grafted Liposomes: In Vitro Studies. *Bioconjug Chem.* **1999**, *10*, 289–298. https://doi.org/10.1021/BC9801124.
132. Zalipsky, S.; Mullah, N.; Harding, J.A.; Gittelman, J.; Guo, L.; DeFrees, S.A. Poly(ethylene glycol)-Grafted Liposomes with Oligopeptide or Oligosaccharide Ligands Appended to the Termini of the Polymer Chains. *Bioconjug Chem.* **1997**, *8*, 111–118. https://doi.org/10.1021/BC9600832.
133. Ishida, T.; Iden, D.L.; Allen, T.M. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. *FEBS Lett.* **1999**, *460*, 129–133. https://doi.org/10.1016/S0014-5793(99)01320-4.
134. Mamot, C.; Drummond, D.C.; Noble, C.O.; Kallab, V.; Guo, Z.; Hong, K.; Kirpotin, D.B.; Park, J.W. Epidermal Growth Factor Receptor–Targeted Immunoliposomes Significantly Enhance the Efficacy of Multiple Anticancer Drugs In vivo. *Cancer Res.* **2005**, *65*, 11631–11638. https://doi.org/10.1158/0008-5472.CAN-05-1093.
135. Kirpotin, D.B.; Drummond, D.C.; Shao, Y.; Shalaby, M.R.; Hong, K.; Nielsen, U.B.; Marks, J.D.; Benz, C.C.; Park, J.W. Antibody Targeting of Long-Circulating Lipidic Nanoparticles Does Not Increase Tumor Localization but Does Increase Internalization in Animal Models. *Cancer Res.* **2006**, *66*, 6732–6740. https://doi.org/10.1158/0008-5472.CAN-05-4199.
136. Guan, J.; Shen, Q.; Zhang, Z.; Jiang, Z.; Yang, Y.; Lou, M.; Qian, J.; Lu, W.; Zhan, C. Enhanced immunocompatibility of ligand-targeted liposomes by attenuating natural IgM absorption. *Nat. Commun.* **2018**, *9*, 2982. https://doi.org/10.1038/s41467-018-05384-1.
137. Munster, P.; Krop, I.E.; LoRusso, P.; Ma, C.; Siegel, B.A.; Shields, A.F.; Molnár, I.; Wickham, T.J.; Reynolds, J.; Campbell, K.; et al. Safety and pharmacokinetics of MM-302, a HER2-targeted antibody–liposomal doxorubicin conjugate, in patients with advanced HER2-positive breast cancer: A phase 1 dose-escalation study. *Br. J. Cancer* **2018**, *119*, 1086–1093. https://doi.org/10.1038/s41416-018-0235-2.
138. Gabizon, A.; Catane, R.; Uziely, B.; Kaufman, B.; Safra, T.; Cohen, R.; Martin, F.; Huang, A.; Barenholz, Y. Prolonged Circulation Time and Enhanced Accumulation in Malignant Exudates of Doxorubicin Encapsulated in Polyethylene-glycol Coated Liposomes 1 [Online]. *Cancer Res.* **1994**, *54*, 987–992. Available online: https://pubmed.ncbi.nlm.nih.gov/8313389/ (accessed on 26 October 2026).
139. Wang-Gillam, A.; Li, C.P.; Bodoky, G.; Dean, A.; Shan, Y.S.; Jameson, G.; MacArulla, T.; Lee, K.H.; Cunningham, D.; Blanc, J.F.; et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): A global, randomised, open-label, phase 3 trial. *Lancet* **2016**, *387*, 545–557. https://doi.org/10.1016/S0140-6736(15)00986-1.

140. Lancet, J.E.; Uy, G.L.; Cortes, J.E.; Newell, L.F.; Lin, T.L.; Ritchie, E.K.; Stuart, R.K.; Strickland, S.A.; Hogge, D.; Solomon, S.R.; et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. *J. Clin. Oncol.* **2018**, *36*, 2684–2692. https://doi.org/10.1200/JCO.2017.77.6112.
141. Griffith, D.E.; Eagle, G.; Thomson, R.; Aksamit, T.R.; Hasegawa, N.; Morimoto, K.; Addrizzo-Harris, D.J.; O'Donnell, A.E.; Marras, T.K.; Flume, P.A.; et al. Amikacin Liposome Inhalation Suspension for Treatment-Refractory Lung Disease Caused by Mycobacterium avium Complex (CONVERT). A Prospective, Open-Label, Randomized Study. *Am. J. Respir. Crit. Care Med.* **2018**, *198*, 1559–1569. https://doi.org/10.1164/RCCM.201807-1318OC.
142. Tak, W.Y.; Lin, S.M.; Wang, Y.; Zheng, J.; Vecchione, A.; Park, S.Y.; Chen, M.H.; Wong, S.; Xu, R.; Peng, C.Y.; et al. Phase III HEAT study adding lyso-thermosensitive liposomal doxorubicin to radiofrequency ablation in patients with unresectable hepatocellular carcinoma lesions. *Clin. Cancer Res.* **2018**, *24*, 73–83. https://doi.org/10.1158/1078-0432.ccr-16-2433.
143. Löhr, J.M.; Haas, S.L.; Bechstein, W.O.; Bodoky, G.; Cwiertka, K.; Fischbach, W.; Fölsch, U.R.; Jäger, D.; Osinsky, D.; Prausova, J.; et al. Cationic liposomal paclitaxel plus gemcitabine or gemcitabine alone in patients with advanced pancreatic cancer: A randomized controlled phase II trial. *Ann. Oncol.* **2012**, *23*, 1214–1222. https://doi.org/10.1093/ANNONC/MDR379.
144. Senzer, N.; Nemunaitis, J.; Nemunaitis, D.; Bedell, C.; Edelman, G.; Barve, M.; Nunan, R.; Pirollo, K.F.; Rait, A.; Chang, E.H. Phase I Study of a Systemically Delivered p53 Nanoparticle in Advanced Solid Tumors. *Mol. Ther.* **2013**, *21*, 1096–1103. https://doi.org/10.1038/MT.2013.32.
145. Barenholz, Y. Doxil®—The first FDA-approved nano-drug: Lessons learned. *J. Control. Release* **2012**, *160*, 117–134. https://doi.org/10.1016/j.jconrel.2012.03.020.
146. Meyerhoff, A.U.S. Food and Drug Administration Approval of AmBisome (Liposomal Amphotericin B) for Treatment of Visceral Leishmaniasis. *Clin. Infect. Dis.* **1999**, *28*, 42–48. https://doi.org/10.1086/515085.
147. Barbazetto, I.; Burdan, A.; Bressler, N.M.; Bressler, S.B.; Haynes, L.; Kapetanios, A.D.; Lukas, J.; Olsen, K.; Potter, M.; Reaves, A.; et al. Photodynamic therapy of subfoveal choroidal neovascularization with verteporfin: Fluorescein angiographic guidelines for evaluation and treatment--TAP and VIP report No. 2. *Arch. Ophthalmol.* **2003**, *121*, 1253–1268. https://doi.org/10.1001/archopht.121.9.1253.
148. Batist, G.; Ramakrishnan, G.; Rao, C.S.; Chandrasekharan, A.; Gutheil, J.; Guthrie, T.; Shah, P.; Khojasteh, A.; Nair, M.K.; Hoelzer, K.; et al. Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. *J. Clin. Oncol.* **2001**, *19*, 1444–1454. https://doi.org/10.1200/JCO.2001.19.5.1444.
149. Gorfine, S.R.; Onel, E.; Patou, G.; Krivokapic, Z.V. Bupivacaine extended-release liposome injection for prolonged postsurgical analgesia in patients undergoing hemorrhoidectomy: A multicenter, randomized, double-blind, placebo-controlled trial. *Dis. Colon Rectum* **2011**, *54*, 1552–1559. https://doi.org/10.1097/DCR.0B013E318232D4C1.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.