Methods of Manufacture of Lipid Nanoparticles

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Lipid nanoparticles (LNPs) have gained prominence as primary carriers for delivering a diverse array of therapeutic agents. Biological products have achieved a solid presence in clinical settings, and the anticipation of creating novel variants is increasing. These products predominantly encompass therapeutic proteins, nucleic acids and messenger RNA. The advancement of efficient LNP-based delivery systems for biologics that can overcome their limitations remains a highly favorable formulation strategy.

Keywords: lipid nanoparticles ; biologics ; cationic lipid ; liposome ; nucleic acids

1. Introduction

The methods used for formulating LNPs vary from low-energy methods that utilize the thermodynamic, solubility, temperature-dependent properties of the excipients, and high-energy methods that involve high pressure, shear and/or ultrasonic energy input to form nanoparticles from an initially coarsely dispersed mixture of the formulation ingredients. Briefly, the methods of preparation can be classified into (i) high energy (ii) low energy and (iii) organic solvent-based methods.

2. High-Energy Methods

2.1. High-Pressure Homogenization (HPH) Technique

HPH is renowned for its reliability and well-established technique to facilitate large-scale production [1][2]. This method involves using high-pressure homogenizers to force a liquid through a micron-sized gap under high pressure (1×10^7–2×10^8 Pascal). The high pressure causes the liquid to accelerate to a velocity exceeding 277.778 m/s, which generates shear stress and cavitation forces that break down the particles [2]. The quality attributes of nanoparticles is influenced by several factors such as the duration of homogenization, temperature controls, homogenization speed, concentration and the type of surfactant, lipid and drug used. Among these, the drug loading capacity is considerably influenced by Drug's solubility in lipids. The process can be carried out either at a high temperature (hot homogenization) or at below room temperature (cold homogenization) [3].

During the hot homogenization method, the process is performed at temperatures higher (278.15–283.15 kelvin) than the melting point of the lipid. The active ingredient, molten lipid, and the emulsifiers aqueous phase are combined using a high-speed stirrer to form a pre-emulsion. After homogenizing the resulting oil-dispersed-in-water (o/w) emulsion, it is subsequently cooled to room temperature that generates the LNPs. An advantage of this technique is that it allows for the incorporation of lipophilic drugs [4]. Although these offer numerous benefits, it is an energy-intensive process that can expose heat-sensitive compounds to excessive temperatures.

The cold homogenization process involves the incorporation of an active moiety in the molten lipid, which is cooled immediately using liquid nitrogen or dry ice. These can address the shortcomings of hot homogenization, which may result in the separation of lipophilic substances into an aqueous phase, drug degradation, and complexity of crystallization leading to the modification of nanoparticles. Efficient pulverization of lipids is promoted by the increased brittleness resulting from the low temperatures utilized during the process [4].

2.2. Supercritical Fluid Technology (SCF)

SCF technology offers a promising approach for producing nanoparticles, providing several benefits such as precise control over particle size, consistent size distribution and the removal of solvents [5][6]. SCF technology takes a supercritical form and can have its solvent power modified by changing the pressure and temperature [7]. In brief, supercritical carbon dioxide ($scCO_2$) acts as a solvent and the solubilities of solid lipids and drugs are enhanced in $scCO_2$

upon being pumped into the high-pressure vessel. Subsequently, the solid lipids and drugs are subjected to a depressurization process, causing them to become supersaturated and precipitated out, which forms drug loaded LNPs [8]. SCF technology offers a significant advantage in the preparation of LNPs by enabling a substantial increase in the solubility of a substance in the liquid through small pressure changes. In a typical process, controlled depressurization of an SCF can promptly generate the supersaturation of dissolved compounds, leading to their precipitation, although SCF technologies provide advantages in the production of LNPs, the high cost of SCF equipment could be a limiting factor. However, utilizing precise computational modeling of the SCF manufacturing process could be a valuable tool for enhancing these technologies [9].

2.3. Ultrasonication

The principle behind this method involves the utilization of sound waves to decrease the size of particles. This method is dispersion-based and includes melting the lipid matrix along with the drug at a temperature of 278.15–283.15 kelvin above its melting point. After melting, the lipid is dispersed in an aqueous phase that contains a surfactant, with rapid stirring to generate an emulsion. Ultrasound is applied to the entire mixture to minimize the size of droplets and the mixture is subsequently cooled gradually to yield the nanoparticle dispersion. The use of commonly available laboratory equipment is the most prominent advantage of this method, for small-scale production. However, the presence of formulations with a high degree of polydispersity and numerous microparticles can pose challenges for drug delivery approaches based on LNPs [10][11]. This technique is widely used in the preparation of SLNs on a laboratory scale.

2.4. Flash Nanocomplexation (FNC)

Plasmid DNA/polycation nanoparticles have been successfully synthesized using this method, which relies on the complexation of polyelectrolytes in aqueous solutions, protein-loaded chitosan nanoparticles, and protein antigen/oligonucleotide adjuvant co-encapsulated nano-vaccines. The introduction of multiple jets inside the chamber of a multi-inlet vortex mixer (MIVM) generates turbulence, facilitating instantaneous and highly efficient mixing at a molecular level [12]. By maintaining a homogenous mixing condition throughout the mixing chamber, nanoparticle assembly can take place, leading to the generation of uniform nanoparticles without the necessity of post-production processing [13][14]. The optimization of nanoparticle characteristics, such as size and shape, and their impact on nanoparticle transport kinetics, can be achieved by adjusting factors like jet velocity, the concentrations of various components, and the complexation capacity of each polyelectrolyte component. LNPs generated through FNC utilize a TFF (Tangential Flow Filtration) process to purify and concentrate all the LNPs. KrosFlo® Research 2i TFF system (Repligen, Waltham, MA, USA) equipped with a 100 kDa mPES filter (ID 0.5 mm). TFF utilizes diafiltration, a process that involves the selective removal of permeable molecules from a solution as it flows through a hollow ultrafiltration membrane. The application of diafiltration for nanoparticle purification in colloidal dispersions has been well characterized [15]. TFF provides a pressure-driven one-way permeation that enables the purification of a wide range of liposome nanoformulations, achieving high lipid recovery rates of over 98%. It efficiently eliminates non-encapsulated compounds (>95%) and organic solvents (reduction of >95%) within a short period of time. Filtration is performed by directing the media parallel to and through the membrane, effectively preventing the accumulation of molecules on the membrane. This process continues until the entire volume has fluxed.

2. Low-Energy Methods

2.1. Thin-Film Hydration Method

The thin-film hydration method uses organic solvents (dichloromethane, ethanol, chloroform and a methanol–chloroform mixture) to dissolve lipids; subsequently, the organic solvent can be removed through evaporation under vacuum at a temperature of 318.15–333.15 kelvin, resulting in the formation of a thin lipid film. Further, the thin lipid film gets hydrated in aqueous media by continuous agitation for 2 h at a temperature of 333.15–343.15 kelvin, where it swells to produce liposomes with an aggregation of multilamellar vesicles. These multilamellar vesicles can be downsized either by extrusion through a polycarbonate membrane (French pressure cell), using probe sonication, or through a microfluidizer to attain size uniformity, lamellarity, and nanoparticle distributions. The method of incorporating drugs into liposomes depends on the characteristics of the drug. If the drug is lipophilic, it can be dissolved with the lipids during the liposome preparation process. On the other hand, hydrophilic drugs can be incorporated during hydration or by active loading into the liposomes [16].

2.2. Reverse Phase Evaporation Method

The lipids are dissolved in a low-boiling-point organic solvent and subsequently mixed directly with water or a buffer that contains a water-soluble drug. By utilizing a low-pressure rotary evaporator, the organic solvent is evaporated, and the proportions of both phases are reversed, which generates lipid nano-liposomes dispersed within the aqueous phase. Extrusion or sonication methods can be employed to achieve a reduced particle size and promote mono- and polydispersity in preformed LUVs and MLVs [17].

2.3. Detergent Removal Method

The detergent removal method involves hydrating and solubilizing lipids by utilizing a solution of detergents [18]. Mixing causes the detergent to bind with the phospholipids, resulting in the formation of mixed micelles comprising both detergent and lipids. As the detergent is removed in a successive or progressive manner, the mixed micelles become better off in lipids, resulting in the generation of unilamellar vesicles. Sodium cholate, Triton X-100, sodium deoxycholate, and alkyl glycoside are examples of detergents commonly utilized due to their high critical micelle concentration (CMC). The surfactant can be removed through various methods such as a dialysis membrane, size-exclusion chromatography, or adsorption onto lipophilic beads [19].

2.4. Dehydration–Rehydration Method

In this technique, the lipids are dispersed in small quantities into an aqueous phase containing the drug, which is then followed by sonication. Initially, the water molecules are vaporized through a dehydration process using nitrogen, resulting in the formation of a multilayered film that encapsulates the drugs. Subsequently, a hydration step is conducted to create large unilamellar vesicles (LUVs) that contain the drugs [20].

2.5. Microfluidic-Assisted Method

The nanoparticles are formed by a controlled transition from jet to drip, which is achieved with a specific chip geometry when liquid droplets are propelled into a carrier fluid. To prevent the coagulation and separation of NPs, surfactants are employed as stabilizing agents. Microfluidics offers the possibility of the on-chip preparation of liposomes and other lipid-based nanoparticles, allowing for precise control over the mixing of aqueous and organic phases. As a result, liposomes with improved characteristics can be consistently achieved in a more reproducible manner [21].

2.6. Microemulsion

Microemulsions are pseudo-ternary systems composed of oil, water, and surfactant, frequently used in combination with cosurfactants that display distinct physicochemical attributes such as clarity, thermodynamic stability, low viscosity, and an isotropic nature. These microemulsions, characterized by stable single-phase swollen micelles, form spontaneously and possess the advantage of incorporating significant quantities of both lipophilic and hydrophilic drugs [22].

2.7. Double Emulsion

The main purpose of this method is to prepare LNPs that are loaded with hydrophilic drugs as well as various biological molecules such as peptides and insulin [23]. In this technique, the drug is first dissolved in an aqueous phase and then emulsified in the molten lipid along with an emulsifier to stabilize the primary emulsion. This primary emulsion is dispersed into an aqueous phase that consists of a hydrophilic emulsifier. Further, the double emulsion is mixed and subsequently separated by sifting. Physical instability such as particle growth during storage and broad particle size distribution are the significant drawbacks of this technique [24].

2.8. Phase Inversion Technique

The phase inversion method involves mixing of different formulation components like drugs, a lipid matrix, water, and surfactant on a magnetic stirrer under three temperature cycles (333.15–358.15 kelvin). Following this, the mixture is subjected to a sudden temperature change by diluting with cold-distilled water, resulting in the formation of LNPs. This technique offers the benefit of not requiring organic solvents and less exposure to heat. Nonetheless, it can be a time-consuming process. The stability of LNPs following their manufacture relies on the storage temperature relative to their phase inversion temperature and melting/crystallization points [25][26].

3. Organic Solvent-Based Methods

3.1. Solvent Emulsification—Evaporation Technique

In this method, the drug and lipids are solubilized in non-polar organic solvents such as toluene, cyclohexane, dichloromethane, or chloroform. Emulsification is then performed in an aqueous phase with the use of a high-speed homogenizer. Finally, the organic solvent is removed through stirring at room temperature (298.15 kelvin) under reduced pressure (4000–6000 pascal). Mechanical mixing at a reduced pressure and temperature causes the precipitation of lipids, resulting in the generation of lipid nanoparticles [27]. This method is appropriate for encapsulating heat-labile drugs. Nevertheless, it may be challenging to completely remove organic solvents, especially if the lipids are not very soluble in the solvent. This can potentially lead to toxicity by residual solvents.

3.2. Solvent Emulsification—Diffusion Technique

This approach involves emulsifying the lipid matrix in water under reduced pressure. The lipids are precipitated in an aqueous environment, resulting in a dispersion of nanoparticles with an average diameter ranging from 30 to 100 nm. The solvent of choice such as butyl acetate, ethyl acetate, benzyl alcohol, isopropyl acetate, or methyl acetate must have partial miscibility with water. Since this method does not necessitate the use of heat, it is suitable for thermolabile drugs [28][29].

3.3. Solvent Injection

This technique involves dissolving the lipid matrix in a water-miscible solvent and rapidly injecting the resulting mixture through a needle into a mixed aqueous phase that includes the surfactant, or without surfactant under constant stirring, and the dispersion is filtered. In this method, the process parameters for synthesizing nanoparticles encompass the properties of the injected solvent, the concentration of the lipid, the amount of the injected lipid solution, and the viscosity, as well as the diffusion of the lipid solvent phase into the aqueous phase. This technique offers several advantages, including simple handling procedures and a rapid production process that does not necessitate the use of sophisticated equipment. However, the utilization of organic solvents is a drawback of this method [30]

3.4. Membrane Contactor Technique

This approach utilizes a modified ethanol injection technique, which involves a pair of pressurized vessels. One vessel holds an organic phase containing lipids, while the other contains an aqueous phase. These two phases are separated by a unique porous glass membrane with specific pore sizes that enable the flow of the organic phase. Polypropylene hollow fibers, often used as the membrane, are preferred due to their capability to accommodate larger surface areas and facilitate uniform fluid flows [31]. The lipid phase is forced through membrane pores at a temperature above the lipid's melting point, resulting in the formation of small droplets. As the aqueous phase circulates within the membrane module, it sweeps away the droplets that are formed at the pore outlets, and lipid nanoparticles are formed by cooling the product at room temperature [32]. The process parameters that influence the generation of lipid nanoparticles include the lipid-phase and aqueous-phase temperature, membrane pore size, lipid-phase pressure, and aqueous-phase crossflow velocity. By maintaining the aqueous phase temperature below the lipid's melting point, the solidification of the lipidic phase in the aqueous phase is instantaneous, leading to the generation of nanoparticles. This method is simple with no significant drawbacks and the size of the nanoparticles can be controlled by utilizing the membranes with varying pore sizes.

4. Large-Scale Production of LNPs

The large-scale production approaches for development of LNPs are essential to establish nanoparticles potential in pharmaceutical applications. High-pressure homogenization is a proven method for scaling up nanoparticle production and has been used since 1950s, when it was used to create parenteral emulsion [33]. Pilot-scale studies have demonstrated that HPH can produce both drug-free and drug-loaded LNPs. A systematic analysis was reported for creating Stavudine-loaded LNPs through the HPH method, starting from a laboratory scale and progressing up to an industrial scale [34]. HPH is a favored technique for producing LNPs due to its ease of scale-up, absence of organic solvents, and shorter production times, which make it a practical and environmentally friendly option for industrial applications. LNP production techniques, including HPH, are often hindered by several common challenges such as drug degradation during the manufacturing process, lipid crystallization, gelation phenomena, supercooled melts and changes in lipid and particle shape. However, it is possible to manage these limitations by carefully analyzing production conditions such as temperature range, shear stress, and light, and by improving the selection of drug carriers, formulation, and drug loading methods [35]. Hot melt extrusion coupled with HPH has the potential to create a scalable process for producing

LNPs [36]. This involves feeding the raw materials into the extruder barrel at a temperature higher than the melting point of the lipids used and further reducing the size of the LNPs by attaching a high-pressure homogenizer to the end of the hot-melt extruder barrel using an insulated connector. The concentration of lipids, screw design, and residence time are the process parameters that have the greatest influence on the size of LNPs among all the parameters studied. Gasco et al. utilized the microemulsion method to fabricate LNPs [13]. In industrial applications, it is common to produce the microemulsion within a temperature-regulated tank and then transfer it to a tank of cold water for precipitation. Both methods mentioned above necessitate a heating process that could result in the degradation of heat-labile drugs. Additionally, the HPH method demands an intensive amount of energy, while the microemulsion method employs surfactants. Bulk nanoprecipitation is a suitable method for producing LNPs on a small scale, but achieving perfect mixing with a short mixing time becomes challenging when scaling up to a larger volume. Hence, to enable large-scale production, various mixing devices such as confined impinging jet mixers, microfluidics, and T-mixers have been developed. The synthesis of LNPs through continuous nanoprecipitation has been made possible with the development of microchannel mixers, but the productivity of the process remains low. Microchannels are channels that contain a cross-junction for both lipids and aqueous solutions, along with a T-shaped junction for the insertion of gas [37]. The formation of a lipid solution using a surfactant and water-based organic solvent simultaneously along the cross-junction into the mainstream is employed to focus flow of liquid. To achieve gas displacement, an inert gas is injected into the microchannel's upward main flow, creating a slug flow of the gas–liquid mixture through the T-shaped junction. This liquid flow-focusing technique, based on hydrodynamic focusing, can produce LNPs with small diameters and narrow size distributions. Static mixers consisting of numerous identical and static elements with intricate structures such as tubes, columns, or reactors have been created for the continuous and large-scale production of LNPs [38]. The size of LNPs was found to be significantly affected by the lipid concentration, with an increase in the lipid concentration leading to an increase in particle size.

5. Post-Production Processing for LNPs

5.1. Sterilization

Autoclaving (steam sterilization), gamma irradiation and filtration are the techniques employed for sterilizing LNPs. Radiation sterilization is considered a recognized method by regulatory agencies to meet the sterility criteria of parenteral products, especially thermosensitive drugs. However, radiation can potentially cause the chemical degradation of lipids as well as actives [39]. Steam sterilization is a widely employed method for sterilizing objects that can withstand high temperatures (394.261–413.706 kelvin) (394.15–413.15 kelvin) and pressure (around 110,316–241,317 pascal) [40]. This method is one of the most effective ways to achieve sterilization as it does not affect mean particle size and zeta potential if exposed for short time. Aseptic manufacturing techniques with sterile filtration are typically necessary for biologic drug substances due to their susceptibility to degradation when exposed to heat, radiation, or chemicals. Sterile filtration techniques have found extensive application in the manufacturing of biotherapeutics, including monoclonal antibodies (mAbs) and recombinant DNA-derived proteins. In the production of biotherapeutics, the application of sterile filtration is essential during the preparation of buffer and cell culture media.

Sterile filters function through the process of normal flow filtration, whereby the membrane effectively retains bacteria, cell debris, and insoluble aggregates. These filters are employed for the removal of bacteria and particles from feedstock solutions, safeguarding downstream units against fouling caused by insoluble materials, and enabling sterile fill operations. Sterile filters typically include filters with pore sizes of 0.1 and 0.2 µm, both meeting precise standards for effectively removing microorganisms. The criteria for sterile filtration using 0.2 µm filters rely on the elimination of 10^7 colony-forming units (CFU) of *Brevimunda diminuta* per square centimeter of membrane surface. Polyethersulfone (PES), polyvinylidene fluoride, nylon, and polypropylene (PP) are among the diverse base polymers used to produce sterile filtration membranes [41]. The sterilization of LNPs presents a major challenge due to the risk of destabilization from conventional methods. For instance, the common use of γ radiation for sterilization can result in lipid oxidation and chain fragmentation, impacting both the stability and efficacy of LNPs. Autoclaving has the potential to initiate phase transitions and thermal stress, resulting in the further destabilization of LNPs. The use of filtration and aseptic processing can induce aggregation or deformation owing to shear stress. In response, various alternative sterilization methods such as UV irradiation, ethylene oxide sterilization, and gentle sterile filtration have been investigated as potential solutions. Therefore, the importance of choosing an adequate sterilization method persists to uphold the stability and effectiveness of LNPs throughout industrial production [2].

5.2. Lyophilization

The lyophilization process of the synthesized LNPs is crucial as it ensures chemical and physical stability in long-term storage, which is a critical factor especially for products containing hydrolyzable drugs. The process of lyophilization involves freezing the material, followed by the application of reduced pressure and heat to enable the frozen water within the material to sublimate. Lyophilization conditions, although effective in removing water, can promote the aggregation of lipid nanoparticles, but the use of an appropriate amount of lyoprotectants can minimize this effect [42].

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