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A comprehensive review on novel liposomal methodologies, commercial formulations and *In-Vitro* characterization techniques

[1]Arpita Trivedi, [2*]Ujwal Havelikar, [3]Pallavi Nikhade, [4]Arindam Kolay, [5]Kabhirdas Ghorpade

[1]Student of Doctor of Pharmacy, Department of Pharmacy Practices, NIMS Institute of Pharmacy, NIMS University Jaipur (Rajasthan), India 303121.

[2,4]Assistant Professor, Department of Pharmaceutics, NIMS Institute of Pharmacy, NIMS University Jaipur (Rajasthan), India 303121.

[3]Assistant Professor, Department of Pharmaceutics, Shri. M. S. Kowase College of Pharmacy, Murkhala Gadchiroli (Maharashtra) India, 442605.

[5]Research Scholar, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research, (NIPER) Mohali, (Panjab) India, 160062.

Email – [1] arpitatrivedi2004@gmail.com, 2 havelikar.ujwal@nimsuniversity.org,

Abstract: *Due to their great biocompatibility, biodegradability, and minimal immunogenicity, liposomes are presently the most widely employed nanocarriers for a variety of hydrophobic and hydrophilic compounds that may be biologically active. Additionally, liposomes have demonstrated improved drug solubility and regulated drug delivery, as well as the ability to modify their surface for targeted, extended, and sustained release. Liposomes can be thought of as having developed from traditional, long-circulating, targeted, and immunological liposomes to stimuli sensitive and actively targeted liposomes based on their composition. More liposomes have progressed stages in clinical trials, and several liposomal-based drug delivery systems are presently clinically licensed to treat a number of disorders, including cancer, fungal infections, and viral infections. This study discusses the composition, production procedures, and clinical uses of liposomes.*

Key Words: *Liposomes, Drug formulation, Drug delivery, Methods of preparation & In-vitro characterization techniques.*

1. INTRODUCTION:

The first microencapsulated medications appeared in the 1950s, and soon after that, polymer-based slow-release systems. Soon after, Bangham and colleagues found liposomes phospholipid bilayer nano containers with spherical shape properties as a promising drug delivery mechanism. [1] The swelling phospholipid system was initially described in a 1965 publication. Several encapsulated phospholipid bilayer structures made out of a single bilayer were originally referred to as bingo some's and subsequently as liposomes within a few years. [2] In 1990, Ireland licenses medications that include liposomes and amphotericin B. The American Food and Drug Administration authorised Liposorb Doxorubicin in 1995. In the 1960s, it was generally recognized that tiny lipid vesicles called liposomes could be utilized to encapsulate medications and colours for systemic delivery and medication targeting. [3] The size of a liposomes might range from 20 nm to several micrometres. Due to the amphiphilic properties of lipids, liposomes become desirable candidates for drug cell delivery. Every amphiphilic substance has a component that is soluble in both polar and nonpolar solvents. [4] Polar lipids (lecithin's, cephalism) and soaps, as well as detergents, are examples of amphiphiles that can be included in aqueous mixtures. [8] They create a lipid-crystalline phase at high concentrations that is long-range ordered and may be diluted in excess water to form rather stable colloidal particles [5]. The composition of liposomes may be changed to contain a sterol (such as cholesterol), which helps maintain the fluidity and stability of the bilayer, as well as charged amphiphiles, which can either positively or negatively charge the bilayer. [9] Physical characteristics of liposomes, such as vesicle size and surface charge, lipid composition, and bilayer fluidity, all have a significant impact on how effectively the medications they transport perform pharmacologically. [6]

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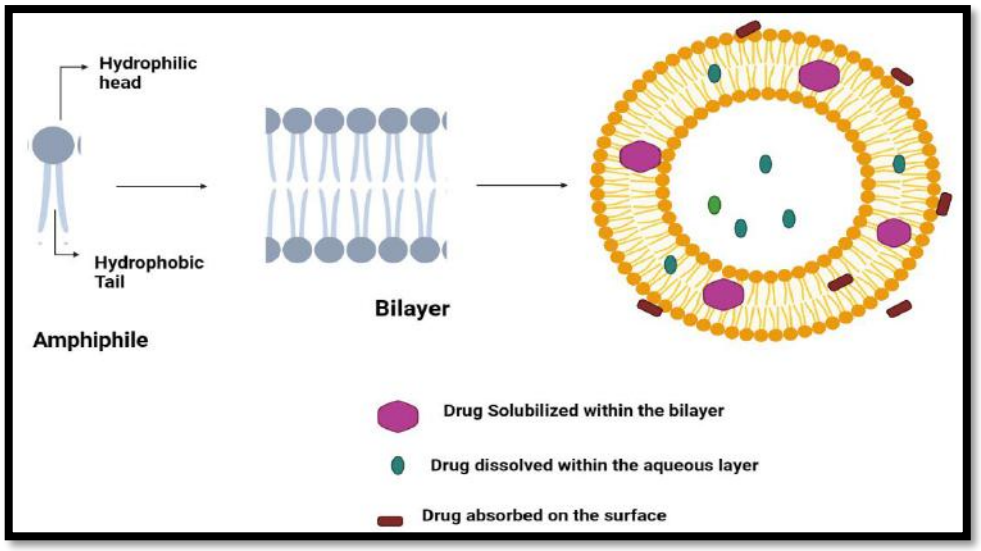

Figure no: - 01 Schematic representation of Liposomes

They have the ability to move their contents both inside cells and to other cell compartments. Water-soluble drugs can be trapped in the inner water region of liposomes whereas lipophilic compounds can be incorporated into the liposomal membrane. [7] There has been an upsurge in the use of liposomes in therapeutics as a means of delivering medications, genes, and vaccines because of its many customizable properties, such as different sizes, charges, and drug retention that may be made for a particular medication and target site.

1.1 ADVANTAGES OF LIPOSOMES

a) Flexibility in the structure in entrapment of water-soluble as well as insoluble drugs.
b) Biodegradability.
c) Efficient control of release.
d) Resemblance to natural membrane structures.
e) Increased targeting prospects.
f) Biocompatible, completely biodegradable, non-toxic, flexible and no immunogenic.
g) Liposomes supply both a lipophilic and aqueous environment in one system.
h) Reduce exposure of sensitive tissue to toxic drugs.
i) Easy to construct.
j) Increases efficacy and therapeutic index.
k) Does not accumulate in the heart and so there is no cardiotoxicity.
l) Prevent oxidation of the drug.

1.2 DISADVANTAGES OF LIPOSOMES

a) Production cost is high.
b) Leakage and fusion of encapsulated drug/molecules.
c) Sometimes phospholipid undergoes oxidation and hydrolysis reaction.
d) Short half-life.
e) Low solubility.
f) They are prone to degradation by oxidation and hydrolysis.

2. IDEAL CHARACTERISTICS OF LIPOSOMES

a) Should have acceptable biocompatibility and toxicity profiles.
b) Should be free from harmful immune or inflammatory reaction.
c) Degradation products should be non-toxic to cells and tissue.
d) Should protect the active components from tensile forces.
e) Should match with the implementation site
f) Should be stable in a physiological environment.
g) Stability should be retaining for a longer duration of the period.
h) Should maintain physical, chemical and biological activity within the body.
i) Should be optimum to prevent dose dumping.
j) Should have maximum loading capacity.

k) Possess relatively easy process availability.
l) Easy malleability into the desired shape.
m) Should be capable of producing a sterile product.
n) Should have a reproducible microscopic and macroscopic structure.
o) Should be similar to the native extracellular matrix.
p) Should have adequate porosity, pore size distribution, and interconnectivity.
q) Should have a high degree of porosity for ideal drug release as well as interaction with organs.

3. METHODS USED IN THE PREPARATION OF LIPOSOMES

a) Thin film hydration (Bangham methods)
b) Solvent Injection methods
c) Reverse phase evaporation
d) Detergent removal methods
e) Dehydration and rehydration method
f) Heating Method
g) pH Jumping Methods
h) Microfluidic Channel method
i) Supercritical fluidic method

A. Thin Film Hydration (Bengham Method):

This approach uses a round-bottom flask to dissolve all lipids as well as the hydrophobic medication in an appropriate organic solvent [10]. After that, a thin film layer was created by the organic solvent gradually evaporating under decreasing pressure [11]. The created thin film is subsequently hydrated with an aqueous buffer solution at a temperature above the lipid's transition temperature (Tm). A hydrophilic medication or drugs may be included in the hydration solution that will be added to the aqueous core of the liposomes. The effectiveness of medication encapsulation depends on the pace of hydration. [12] Wherein the higher the encapsulation effectiveness, the slower the rate of hydration [13]. The extrusion of liposomes via polycarbonate membranes with predetermined hole diameters or the use of a bath or probe sonicator can change the size of the liposomes, the kind of lamellarity, and the dispersion of the particles. Over sonication, the extrusion approach provides more effective encapsulation and stable liposomes. In addition to creating SUV liposomes, sonication can destroy or hydrolyze medicines and/or lipids that are encapsulated. Liposome suspensions may become potentially contaminated with metal as a result of probe sonication (Figure 2) [14].

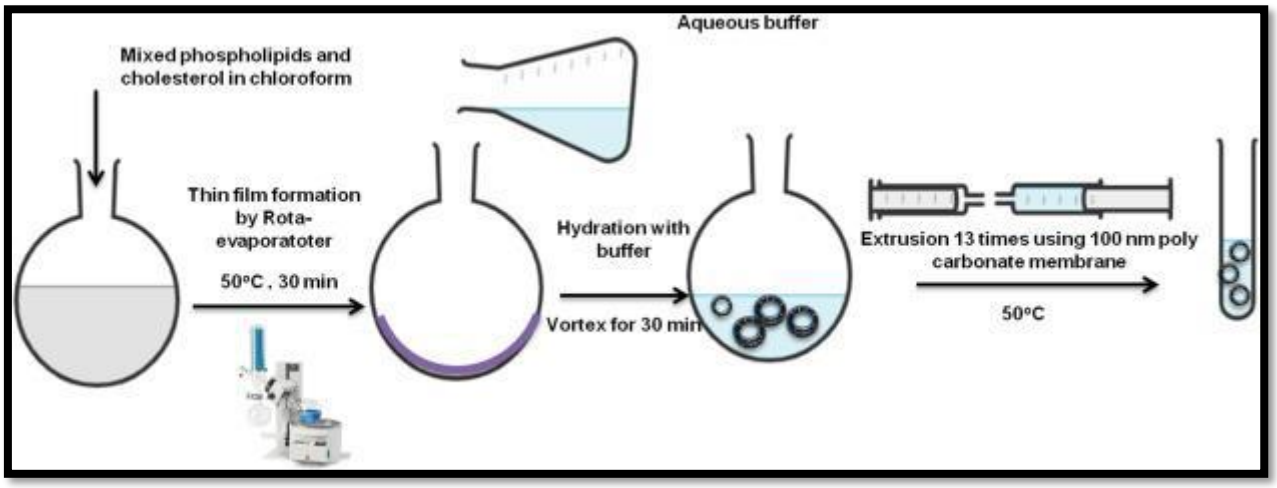

Figure.02 Liposomes preparation *via* thin-film hydration extrusion technique

b. Solvent Injection methods:

According to the kind of organic solvent utilized, the injection techniques were categorized (Figure 3) [15]. The hydrophobic active substances and lipids were quickly dissolved by an organic solvent and introduced into an aqueous phase. Diethyl ether allows for direct solvent evaporation during mixing at a temperature higher than the solvent's boiling point [16]. A 10- to 20-fold aqueous solution was needed to use ethanol for injection, and it can be evaporated under vacuum using a rotary evaporator, dialysis, or filtration. Most liposomal formulations with greater polydispersity indices (PDI) were created using this technique [17]. Continuous exposure to high temperatures and organic solvents may also impair the stability of drugs and lipids [18].

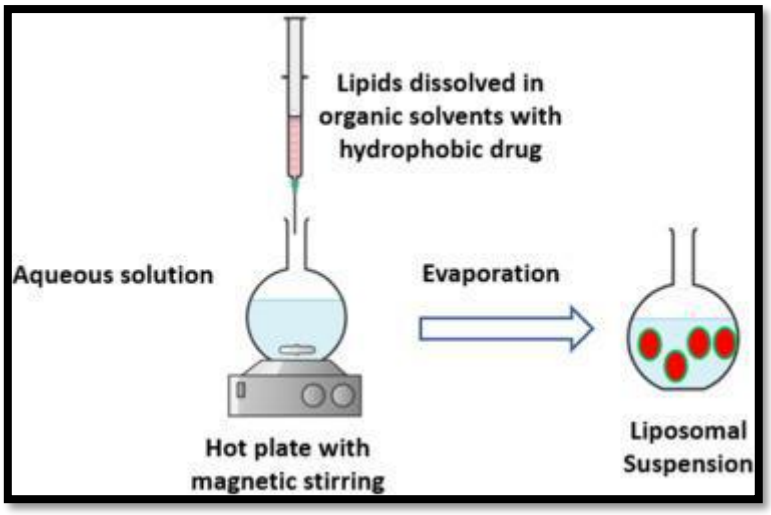

Figure.03 Liposomes preparation *via* Solvent Injection methods

c. Reverse phase evaporation

By stabilizing loaded payloads, overcoming barriers to cellular and tissue absorption, and improving biodistribution of payloads to target areas in vivo, liposomes exhibit enhanced treatments for a variety of biological applications. The first method for creating liposomes to be reported was the Bangham method, also known as thin lipid film hydration technology. The disadvantages of this approach include the potential of high temperatures causing damage to phospholipids and drugs as well as deceased encapsulation and sonicator interaction with the liposomes. The reverse-phase evaporation method, which tends to create inverted micelles or water-in-oil emulsions, is a commonly used preparative option. The medicine is carried by the water phase, whereas the organic phase is composed of the lipids needed to create the liposome bilayer. The solvents used to dissolve the lipid combination are then evaporated. After evaporation, the newly formed lipid film is redissolved in an organic phase. Under low pressure, the organic solvent can slowly evaporate, first turning the dispersion into a viscous gel and then creating an aqueous suspension with the liposomes still in it. Similar to previous preparation techniques, several extrusions through a polycarbonate membrane are required to minimize the size of the liposome produced by the reverse-phase evaporation approach. The volume of the polycarbonate membrane pore and the quantity of extrusion cycles affect the degree of size reduction as well as the final particle size and distribution. In this study, the production method and formation theory of several payload-loaded liposomes via reverse-phase evaporation techniques were summarized.

d. Detergent removal method

In this procedure, a suitable organic solvent was used to dissolve lipids together with a high critical micelle concentration (CMC) surfactant in a round bottom flask. After mild solvent evaporation, a thin coating was produced at the flask's bottom [19]. The lipid film was subsequently hydrated in an aqueous solution that contained drug molecules to produce a mixed micelles solution [20]. The surfactant is subsequently eliminated through dilution, size-exclusion chromatography, adsorption onto hydrophobic beads, dialysis, or any combination of these [21, 22, 23, 24]. After solution concentration, a LUVs liposomes vesicle will be created [25]. The separation of the majority of hydrophilic medications from the liposomes during the detergent removal stage is a major flaw in this approach [26].

e. Dehydration and rehydration method

It is an organic solvent free method to produce LUVs using sonication. This method based on direct dispersing of the lipids at low concentrations into an aqueous solution containing the drug molecules followed by sonication [26]. First, the dehydration step to evaporate water under nitrogen to create multi-layered film entrapping the drug molecules. Then, a hydration step to form large vesicles encapsulating the drug molecules [24]. This method is simple but with high heterogeneity of the liposomes sizes.

f. Heating Method

Additionally, it is a method devoid of organic solvents. This approach involves directly hydrating lipids with aqueous solution and heating them for at least an hour beyond the melting point of the phospholipids being utilized, all while having a hydrating agent present at a concentration of 3-5%, such as glycerine or propylene glycol. When adding cholesterol to the recipe, the suspension can be heated up to 100 °C [27]. The hydrating substances function as stabilizers and isochronizing additives to stop the coagulation and sedimentation of nanoparticles. The heating approach is effective for the production of powder inhalable liposomes because the hydration agents have a cryoprotective effect [28].

g. pH Jumping Methods

The pH jumping method is another solvent-free approach to making liposomes. In this approach, MLVs are converted into SUVs by exposing an aqueous solution of phosphatidic acid and phosphatidyl choline to a nearly four-fold rise in pH during a brief period of time [29, 30]. The proportion of SUVs vs LUVs formed is determined by the ratio of phosphatidic acid to phosphatidyl choline [31].

h. Microfluidic channel method

A brand-new technique for creating liposomes has just been presented, and it uses microfluidic channels (fig.04). Liquids may be used in tiny channels thanks to microfluidic technology [32]. In this technique, lipids are dissolved in ethanol or isopropanol, and the resulting solution is injected into the micro-channels either vertically or in the opposite direction to the aqueous media. This technique produces liposomes by continuously axially combining the organic and aqueous solutions. Surfactants are used to stabilize liposomes in order to prevent coagulation and separation [33]. To produce repeatable liposomes with the required average size, polydispersity, morphology, and lamellarity, microfluidic channel approaches manage the mixing process of the organic and aqueous phases [34].

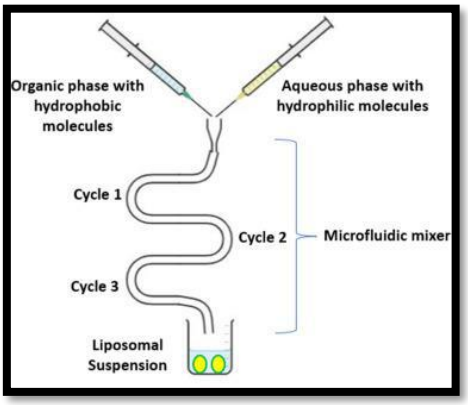

Figure 04. Schematic representation of injection methods method.

3.1 POST PREPARATION HANDLINGS

A) Freeze – thaw cycles

B) Freeze – drying (Lyophilization)

A. Freeze-thaw cycles

This method is typically applied while making liposomes in order to improve lamellarity and encapsulation effectiveness. This method involved performing freeze-thaw cycles between liquid nitrogen temperatures of -196 °C and below the transition temperature of the phospholipids that were being employed [35, 36].

B. Freeze - drying (lyophilization)

The liposomal goods are given this treatment to maintain them and increase their shelf stability. After combining the liposome solution with a cryoprotective, primarily 5–10% sucrose or trehalose, freeze-drying entails deep freezing the mixture [37]. The liquid samples were then transformed into fluffy solid particles by a sublimation stage that was performed at a very low temperature and under decreased vacuum. For liposomes containing thermosensitive biomolecules, lyophilization becomes a necessary treatment [38].

LIPOSOMES	CHARACTERISTICS CHARACTERIZATION TECHNIQUE	REFERENCES
Average particle size	Dynamic light scattering (DLS) and microscope technology: Scanning and transmission electron microscopy (SEM/TEM), cryogenic-TEM (Cryo-TEM), and atomic force microscopy (AFM)	[39, 40]
Zeta potential/Surface charge	Electrophoretic mobility, DLS	[41]
Particle shape/morphology	TEM, Cryo-TEM, and AFM	[42]

Lamellarity	Cryo-TEM and 31P-NMR	[42]
Phase behavior	X-ray diffraction (XRD), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)	[43, 44]
Encapsulation efficiency/Drug release	Centrifugation, dialysis followed by drug content determination using chromatographic and/or spectrophotometric methods	[45, 46]

Table No 01 - Represent different techniques used for the assessment of liposome parameters.

3.2 LIPOSOME CHARACTERIZATION TECHNIQUES

- **Liposomes morphology identified by microscope techniques:**

Several TEM methods, such as the negative stain, freeze structure, and cryogenic microscopy, can directly see particle shape and size. In negative-stain TEM, samples are placed on a copper grid covered with a carbon sheet, which is then stained with uranyl acetate or phosphotungstic acid to produce enough contrast when exposed to high-energy electron beams. Faux staining is a method used to loosely verify nanoparticle sizes, and it typically takes less than an hour. However, because samples must be dried in the air before staining, these liposomes may contract, collapse, or assemble on the grid during the staining procedure. No drying procedures are needed with the freeze structure method. Instead, it quickly freezes the sample to vitrify it, then breaks apart the frozen sample to reveal a surface plane. The development of cryo-TEM later allowed for the direct verification of a thin aqueous film for imaging, showing high-resolution morphology and intricate structural details concerning lipid layer and encapsulating processes. Due to the destructive sample preparation steps of drying or fixing the sample, followed by imaging under high vacuum, scanning electron microscopy (SEM), which uses an electron beam to scan across the sample surface rather than penetrate through the sample material as TEM, is not frequently used for imaging of lipid particles. The environmental scanning electron microscopy (ESEM) technique has recently been created to examine materials in their hydrated states and to look into how samples' states change dynamically in various surroundings. Additionally, the three dimensional structure of liposomes might be studied using AFM, which analyses interactions between the sample surface and a probing tip. According to Robson et al. [50], several imaging approaches for liposomes have been thoroughly compared.

- **Liposomes size determined by light scattering techniques**

Although microscopes are excellent tools for determining particle morphology and structure, they can only provide a semi-quantitative analysis of particle size distributions from representative images and may not be able to reveal extremely small particles or extremely large aggregates, especially if there aren't many of either. DLS, on the other hand, has established itself as the standard method for quick and accurate study of nanoparticle size distributions with diameters between 0.5 and 1000 nm. DLS tracks time-varying variations in the light dispersed by particles moving in Brownian motions [51]. Temperature, solvent viscosity, and solvent refractive index are three sample factors that might vary during DLS tests and should all be predetermined in order to establish the hydrodynamic particle size Rh precisely.

1) The hydrodynamic particle radius is calculated using the assumption that particles are spheres.

(2) The wavelength of the light source, which is normally set at a high value, such as 633 nm or 830 nm, should not be absorbed, illuminated, or fluoresced by the samples.

(3) Sample dilutions are frequently necessary to prevent particle-particle interactions and multiple scattering, which have an impact on both viscosity and diffusion. Therefore, it is anticipated that particles would maintain their form and size against dilutions.

(4) Before securely publishing any Rh data, one should always examine the autocorrelation curves' fitting quality, and a multimodal sample should only be reported for individual size peaks;

(5) Because big particles scatter more light than their smaller ones, the intensity-derived Rh is substantially heavier for large particles. Particle subpopulations may only be distinguished when their mean sizes differ by at least 2-3 folds because signals from tiny particles may be hidden by big particles.

- **Liposomes size measured by size exclusion chromatography (SEC)**

Using their varied sizes, SEC distinguishes drug-loaded nanoparticles from free medicines. Analytical SEC may be used to determine particle size distributions using appropriate standards or light scattering detectors, whereas preparative SEC is mostly used for purification/fractionation of nanoparticles or for buffer exchange. Larger particles elute more quickly than smaller ones in an SEC column because matrixes with the right pore size allow eluates of different sizes to penetrate or not permeate into the gel particles. For the separation of nanoparticle populations, a variety of Sepharose (large-pore agarose) and Sephadex (cross-linked dextran's) gels have been utilized, however their exclusion limits (100 nm) prevent the separation of particle populations bigger than 100 nm [19].

- **Liposomes surface charge (zeta potential)**

The head groups of phospholipids, which may include positively charged tertiary amines or negatively charged carboxyl's, often dictate the surface charges of lipid nanoparticles. Because it affects the potency of intra-particle interactions, the adsorption of counter ions, and ultimately particle stability, a nanoparticle's surface potential is a crucial physicochemical property. Zeta potential, which represents the charge profile of a naked particle coupled with a diffusive layer and may be computed from the electrophoretic mobility of particles determined by phase analysis light scattering (PALS) [53, 54], is the most common way to express this parameter. In order to get reliable measurements, it is necessary to predetermine key medium variables such as temperature, viscosity, refractive index, and phase nature. The zeta potential of liposomes did, in fact, appear to be considerably influenced by the pH, temperature, and ionic strength of the medium, according to various findings [27, 28].

- **Stability study**

Physical stability (nanoparticles should retain a homogenous size distribution and a consistent amount of drug encapsulation) and chemical stability (lipids and APIs shouldn't degrade during storage) are also requirements for lipid nanoparticle formulations.

A) Physical stability

During storage, small lipid nanoparticles with moving lipid bilayers may combine to form bigger vesicles. These particles can combine to cause phase separation and the leaking of the encapsulated API before, during, or after lipid fusion. The differential scanning calorimetry (DSC) and fluorescence-based lipid mixing tests have mostly been used to study liposome fusion. According to the DSC method, the union of two populations of liposomes made up of two different kinds of lipids with different transition temperatures would result in the introduction of a new endothermic phase transition peak [54]. Although label-free assessments of liposomal fusion are possible using DSC, this method only works with pure lipid systems with known transition temperatures and requires a lot of liposome samples because of its generally low sensitivity. The fusion process may be simultaneously visualized and quantified using fluorescence-based techniques, which are often based on quenching/sequencing or the Förster resonance energy transfer (FRET) of fluorophore pairings [55]. It is possible to independently encapsulate an aqueous-soluble fluorophore and its corresponding fluorescence enhancer or quencher in the liposome core. Liposomal fusion would then be indicated by changes of the fluorescence intensity upon complexation of the reagents. An aqueous-soluble fluorophore and the accompanying fluorescence enhancer or quencher can both be separately encapsulated in the liposome core. The intensity of the fluorescence would then alter when the reagents were complexed, signalling the fusing of the liposomes. Terbium (Tb) and dipicolinic acid (DPA) were chelated in one method, resulting in the [Tb(DPA)3]3 complex, which increased Tb's fluorescence intensity by four orders of magnitude [36]. In a different method, the fluorescence quenching of amino naphthalene trisulfonic acid (ANTS) by p-xylene-bis (pyridinium) bromide (DPX) was seen in distinct liposomes containing ANTS and DPX. As an alternative, the lipid component can contain two lipophilic fluorophores with overlapping fluorescence spectra.

B) Chemical stability

The encapsulated API can be removed and its chemical stability evaluated, often by LC MS, or its physical states can be determined in situ using SAXS or SANS, as was mentioned before. Lipid degradation, mostly by hydrolysis and/or oxidation, can compromise the stability of lipid bilayers and lipid nanoparticle formulations.

- **In vitro drug release**

Before delivering the medicine to the target, stable nanoparticle formulations should keep the loaded drug during in vitro storage and in vivo circulation. There have been several efforts made to create trustworthy in vitro release testing techniques and forecast in vivo behaviour of nanoparticle formulations [55, 56, 57]. Generally speaking, these techniques may be divided into four groups: sampling and separation (SS), dialysis membrane (DM), continuous flow (CF), or mixed procedures. The SS method, which is the most straightforward, entails incubating samples in the release media, sampling, isolating released API from intact nanoparticles often using stand-alone ultracentrifugation or filtering, and then off-line API measurement. The selection of physiology-relevant release medium and the proper sampling time frame are significant variables. According to the U.S. FDA's draught guidance on liposomal doxorubicin formulations, drug release should be tested in the following conditions (1) 50% human plasma at 37 °C for 24 h to gauge blood circulation stability; (2) various pH buffers (7.5, 6.5, and 5.5) at 37 °C for 24 h to simulate drug release in normal tissues, cancerous tissues, and cells, respectively; (3) elevated temperatures (43, 47, 52, and 57 °C). Acidic pH and/or enzymatic conditions may also be necessary for the release of nucleic acid APIs from complexed lipids. This might resemble endo/liposome settings and connect medication release with intracellular delivery effectiveness [58]. Additionally, it is advised to attain a plateau for the final three sample time points and at least 85% encapsulated drug release [59].

4. SUMMARY OF THE ANALYTICAL CHARACTERIZATION TECHNIQUES :

In order to characterize lipid nanoparticle formulations for distinct CQAs, a variety of analytical techniques have been used to date. Those reviewed in depth in this review are summarized in Table 1. Various fluorometric assays can be used for quantitative analyses of lipid amounts, liposome lamellarity, and liposome fusion, while some of these techniques can be used to investigate multiple attributes. For example, RP-HPLC can be used for separation and quantification of both lipid species and nanoparticles. On the other hand, several analytical options have been established to characterize a certain property. Since each method has unique advantages and disadvantages, it is advisable to choose the most appropriate method based on the specific analytical objective and necessity. For instance, DLS or microscopy techniques could be used to analyses nanoparticle size qualitatively or quantitatively, respectively; LC in conjunction with multiple detectors would enable accurate quantification of lipid species and APIs; and gentle separation techniques like CE or FFF could be used when high sample recovery rate is the main priority.

5. CONCLUSIONS AND FUTURE PERSPECTIVES :

In conclusion, liposomes and other lipid nanoparticles are being used more often to address the delivery issues posed by fresh pharmaceutical delivery systems. It is essential for formulation development, quality assurance, and the clinical translation of novel therapeutics to address CQAs of these nanoparticle formulations using the proper analytical methodologies. We looked at the methods employed to characterize these CQAs, such as lipid and API identification and quantification, particle properties, stability, and drug release. In the early stages of developing nanoparticle formulations, robust and high-throughput manufacturing and analytical procedures would be ideal, taking into account the need to screen and optimize several process factors and formulation features.

6. DECLARATIONS :

6.1 . Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

6.2 Funding statement

This review did not receive any specific funding agencies in the public, commercial, or not-for-profit sectors also institutions or University.

6.3 Declaration of interest's statement

No conflict of interest.

6.4 Acknowledgment

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