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From Bench to Application: Formulation and Evaluation of Liposomes in Drug Delivery

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Abstract:

Liposomes are small, spherical entities consist of cholesterol and phospholipids, delivering substantial benefits. They act as transporters for medications, enabling their precise delivery in various treatment. They offer more advantages than other formulations. They can provide sustained drug delivery, enhance hydrophobic drugs' solubility, and increase their bioavailability. This review explores the pros and cons of liposomes, reviews traditional preparation methods, evaluates assessment techniques, and discusses their applications. There are some stability problems of liposomes through traditional methods of liposome preparation, so to overcome this, many novel approaches are being developed by researchers. This review additionally examines the recently developed innovative techniques for liposome preparation. This review further explores the newly developed innovative methods for the preparation of liposomes. Furthermore, the review offers concise details regarding the classification, structure, and composition of liposomes.

Keywords: Liposomes: Classification, Structural Characteristics, Preparation Techniques, Innovative Preparation Methods, and Evaluation Criteria.

1. Introduction:

Due to numerous benefits and reduced side effects compared to alternative formulations, liposomes are widely utilized for drug delivery. Liposomes are tiny, spherical structures that transport drugs and other substances to cells. Liposomes are a Greek word. 'Lipo' means 'Fats' and 'Somes' means 'body'. These are fundamental microscopic vesicles made up of layered bilayers that enclose a water-filled space within a phospholipid bilayer. They function as a mechanism for delivering both nutrients and medicinal compounds. They serve as a means for delivering nutrients and pharmaceutical medications. Their ability to encapsulate and protect various molecules, combined with their biocompatibility and biodegradability, makes them promising carriers for therapeutics, vaccines, and cosmetics. Liposomes are used in medicine, cosmetics, and other applications. In 1961, Alec D. Bangham discovered liposomes in England, unveiling that phospholipids possess the extraordinary capability to swiftly organize into closed bilayer formations called vesicles in a water-based environment because of their amphiphilic characteristics. [1] Liposomes, varying in size from 5 to 200 nm, are recognized for their capacity to encapsulate not only hydrophilic but also lipophilic drugs within the lipid bilayer or the surrounding aqueous medium, thus establishing a foundation for their utilization in drug delivery systems. [2] Since the commencement

of clinical trials of a liposome-encapsulated medication in 1985, over 40 liposome-based drug delivery formulations have advanced through different clinical phases and have become available in the market. [3]

1.1 Advantages of Liposomes:

1. Liposomes protect the DNA from degradative processes.
2. They deliver hydrophobic drugs.
3. These can be directed toward particular cells or tissues.
4. They safeguard encapsulated drugs from the external environment.
5. These materials are safe, adaptable, biologically compatible, biodegradable, and non-immunogenic.
6. They minimize the toxicity of the encapsulated material.
7. They minimize the contact of delicate tissues with harmful medications.
8. They increase stability via encapsulation. [4, 5, 6]

1.2 Disadvantages of Liposomes:

1. Liposomes have lower solubility and stability.
2. These have shorter half-life.
3. There is a risk of leakage and fusion of encapsulated drugs because phospholipids can sometimes undergo oxidation as well as hydrolysis reactions.
4. The production costs are significant, and there is a risk of allergic reactions to the components of liposomes.
5. Their large size presents challenges in effectively targeting different tissues.
6. The challenge of targeting different tissues arises from their considerable size. [4, 5, 6]

2. Structure of Liposome:

Liposomes consist of a lipid bilayer, typically ranges in diameter from 50 to 1000 nanometers, serving as a targeted delivery mechanism for active biological substances. Cholesterol and phospholipids are the primary components of liposomes consisting of one or more layers encasing a core filled with water. [5] Liposomes have a structure similar to cell membranes.

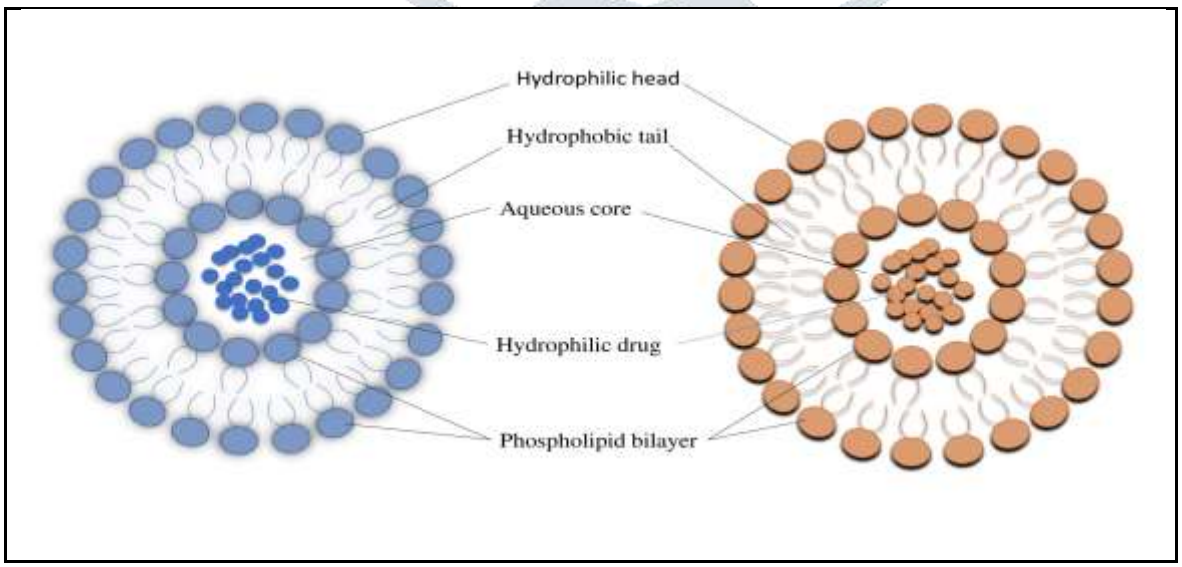

Fig.1: Structure of Liposome

2.1 Components of Liposome:

The components of Liposome are as follows:

1) Phospholipid
2) Cholesterol
3) Polymeric materials
4) Surfactant

1) Phospholipid:

Liposomes predominantly consist of safe phospholipids, which are essential components. These phospholipids have an important role in creating the bilayer membrane which surrounds the liposome's aqueous core, with

hydrophilic heads and lipophilic tails. Hydrophilic component of the phospholipid molecule, which interacts with aqueous environments, typically comprises a phosphate group attached to the glycerol. Lipophilic tails: Phospholipids have hydrophobic tails and hydrophilic heads that create stable bilayers, a barrier for the liposome's aqueous core. The type and composition of phospholipids are vital for liposome stability, affecting size, charge, and degradation susceptibility. [7]

A) Synthetic Phospholipids: These are phospholipids that are prepared by some chemical modifications in natural phospholipids. They enhance the targeted delivery of liposomes. They have high purity, stability, and controlled properties. [8]
Ex: Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) lipids. [8]

B) Natural Phospholipids: Natural phospholipids derived from soybeans as well as egg yolks are essential for pharmaceuticals. Lecithin, a sustainable source rich in phosphatidylcholine (PC), is crucial for liposome formulation. The stability of healthy cell membranes is primarily due to glycerophospholipids. However, natural phospholipids tend to be less stable than synthetic alternatives. [9]
Ex: Egg phosphatidylcholine, Soybean phosphatidylcholine. [9]

C) Sphingomyelins: They are composed of a structural backbone and sphingosine, featuring cis-double bonds connected by amide bonds within the acyl chains, which facilitate both intermolecular and intramolecular hydrogen bonding. Liposomes consist of non-toxic phospholipids, which are crucial for forming the bilayer membrane that surrounds their aqueous core, with hydrophilic heads and lipophilic tails. [7]
Ex: Sphingomyelin and glycosphingolipids. [7]

D) Gangliosides: These molecules found in grey matter contribute slightly to the formation of liposomes. They have complex saccharide structures with sialic acid residues in the polar head group, giving them negative charges at neutral PH. Their presence enhances the charged surface layer of liposomes. [7]
Ex. GM1, GD1a, GD1b, and GT1b. [7]

E) Cationic lipids: Along with DOTAP (Dioleoyl propyl trimethyl ammonium chloride), DODAB, often referred to as C-Dioctadecyl dimethyl ammonium bromide or chloride, is a molecule that functions as an analogue to a number of other chemicals, such as cationic cholesterol derivatives and other DOTMA derivatives. [7, 10]

2) Cholesterol:
Cholesterol is vital for strengthening liposome membranes, increasing rigidity, and reducing permeability. It is added to adjust membrane fluidity and stability by fitting between phospholipids, typically in a 1:1 to 2:1 ratio with phosphatidylcholine. [11] Hydrophobic tails of liposomes are situated within the bilayer while, the hydroxyl groups of cholesterol are oriented towards the water. [6] This incorporation boosts liposome rigidity and decreases hydrophilic substance permeability, enhancing the lipid bilayer's mechanical stability. In contrast, liposomes lacking cholesterol are more fluid and less stable, which may hinder drug delivery effectiveness. [12]

3) Polymeric materials:
Synthetic phospholipids with diacetylene groups polymerize under UV light, forming liposomes with improved permeability for encapsulated drugs. [7]
Ex: Additional polymerizable lipids include those with conjugated dienes, methacrylate, and similar compounds.

4) Surfactant:
Single-chain surfactants, particularly those with cholesterol, are vital in liposome formulations as they decrease surface tension between immiscible phases, influencing entrapment and release, while also enhancing lipid bilayer stability. [9, 13, 14]

3. Method Of Preparation of Liposome:

The overall procedure for preparing liposomes includes several essential stages.

1) Extraction of lipids using an organic solvent.
2) Their subsequent incorporation into a water-based medium.
3) Purification of liposomes.

4) Assessment of the final outcome. [15]

3.1 Conventional Methods:
A. Passive loading techniques:
1. Mechanical dispersion
   a) Thin Lipid Film hydration
   b) Micro-emulsification
   c) Sonication method
   d) French pressure cell
   e) Membrane extrusion
   f) Dried reconstituted vesicles
   g) Freeze-thaw technique
2. Solvent dispersion
   a) Ethanol injection technique
   b) Ether infusion technique
   c) Double emulsification
   d) Reverse-phase evaporation technique
3. Detergent removal
B. Active loading techniques

3.1 Conventional Methods:
A. Passive Loading:
Passive loading enables concurrent creation of liposomes and the inclusion of therapeutics. [16] While hydrophobic molecules are incorporated into the lipid bilayer, hydrophilic substances are found in the water-based surroundings both within and outside the liposomes. Lipids are mixed with water-insoluble medications in a solvent that is organic, which is later evaporated into a hydrated thin film and form liposomes. [16, 17]

1) Lipid film hydration technique:

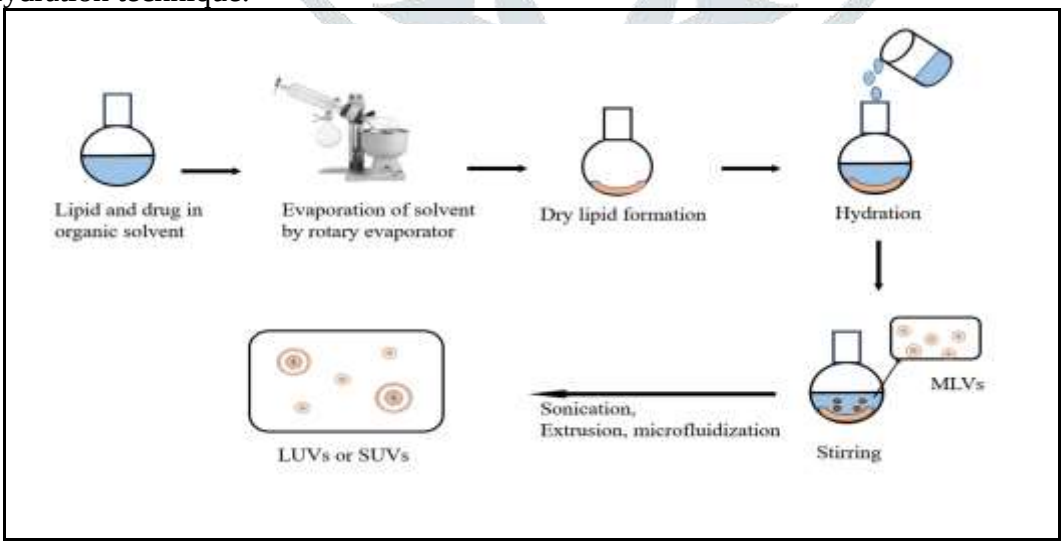

Fig.2 Phases involved in the thin-film hydration technique for liposome synthesis.

Bangham technique is a broadly recognized and straightforward approach for producing liposomes. This technique entails the dissolving lipids in a solvent which is organic such as methanol, ethanol, or chloroform, followed by the evaporation of the solvent to form a thin lipid film, which is subsequently infused with water, typically using a buffer, at 60 to 70 °C for 1 to 2 hours, which is near the lipid's phase transition temperature. Agitating the film helps form liposomes, which are then allowed to rest overnight at 4 °C. During hydration, the lipids absorb water and swell, resulting in a multi-lamellar vesicle (MLV) suspension.[5]

Advantages:
- It is a simple process.
- Widely used method.
- It is applicable for various types of lipid blends.
- Straightforward approach. [5]

Disadvantages:

- Difficulty in scaling up.
- Water-soluble medications exhibit poor entrapment efficiency.
- Challenges in eliminating organic solvents.
- Large vesicles lacking particle size regulation.
- The process is time intensive.
- Sterilization is required. [5]

2) Sonication:

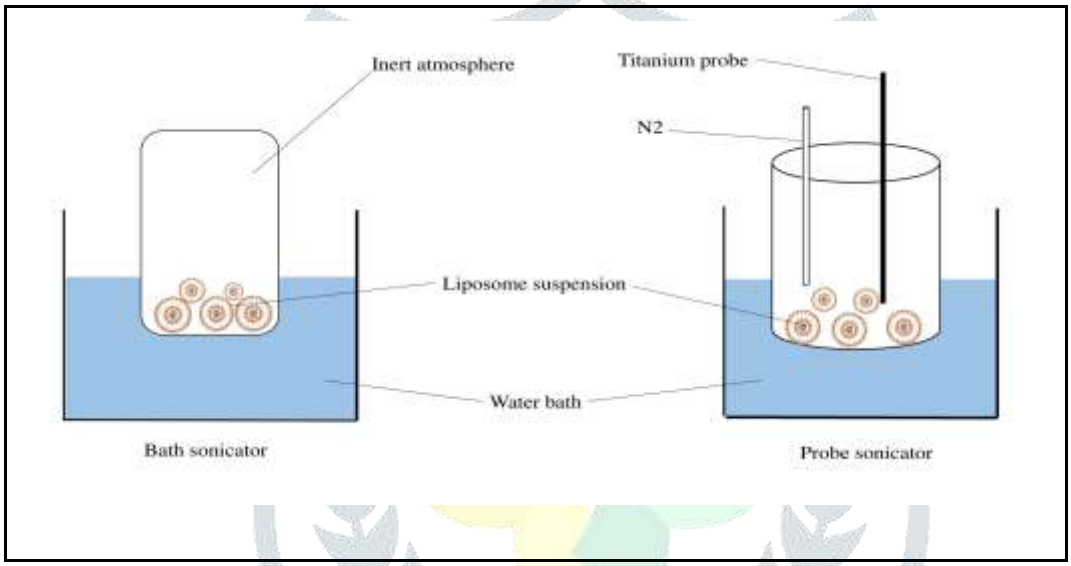

Fig.3: Bath sonicator and Probe sonicator

Sonication serves as the primary method for converting multi-lamellar vesicles (MLVs) into small uni-lamellar vesicles (SUVs), employing either a probe or bath sonicator within a regulated inert atmosphere.

a) Probe sonication: It is used for small volumes. This involves egrassing the sonicator tip in the liposome suspension solution, while a cooling bath composed of water and ice surrounds the vessel to avert the degradation of lipids. [18] However, this method risks contaminating the lipid solution with metal particles from the probe tip and can cause de-esterification of sensitive lipids (≥3%) if sonicated for over an hour. [19, 20]

b) Bath sonication: Bath sonication effectively disperses large liposomes in a sterile, temperature-controlled container, but has limitations including poor encapsulation efficiency, the risk of phospholipid degradation, and significant size polydispersity. It can create small unilamellar vesicles (SUVs) of 15 to 25 µm but may not achieve the desired liposome characteristics. [19, 20]

3) Micro-emulsification:

Small vesicles are produced from concentrated lipid suspensions using a microfluidizer, while large multilamellar vesicles are subjected to high-pressure filtration through a 5-micrometer filter, subsequently passing through narrow microchannels where two streams converge at high velocity. For biological applications, the rotation speed is usually between 20 and 200 rpm, and the fluid can be recirculated until spherical vesicles are formed. [7]

4) French pressure cell method:

This technique produces unilamellar liposomes from multilamellar vesicles (MLVs) by hydrating MLVs in water and applying high pressures of 10,000 to 20,000 psi, forcing them through a narrow opening to create smaller, uniform liposomes that can be collected and purified to eliminate aggregates. [21]

5) Membrane extrusion:

Membrane extrusion employs a filter to generate liposomes of precise dimensions for laboratory research, generating small and large unilamellar vesicles (SUVs and LUVs) from multilamellar vesicles (MLVs). By applying low pressure (under 100 psi), liposomes pass through a polycarbonate membrane, reducing their size. LUVs can be pre-treated with freeze-thaw cycles or filtered through larger pores (0.2 to 1 µm) to disrupt phospholipid bilayers. Increasing transmembrane pressure and extrusion cycles further reduce vesicle size, resulting in liposomes of 120 to 140 nm. [22]

6) Freeze-thaw method:
Small Unilamellar Vesicles freeze quickly and thaw gradually. A short sonication disperses aggregates into large uni-lamellar vesicles. The creation of large uni-lamellar vesicles occurs through the merging of small unilamellar vesicles during freezing or thawing, which is inhibited by high ionic strength and increased phospholipid concentrations. [23, 24]

7) Ethanol injection technique:

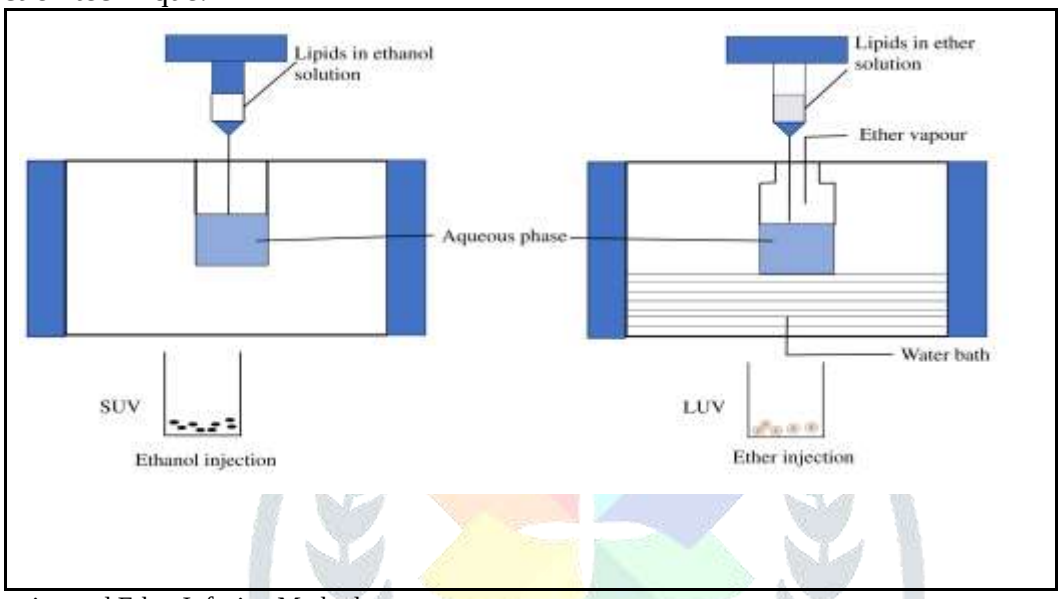

Fig.4: Ethanol Injection and Ether Infusion Method

Ethanol-based lipid solution is rapidly mixed with excess buffer to create multilamellar vesicles (MLVs). However, this approach has drawbacks, including inconsistent liposome size (30 to 110 nm), difficulties in eliminating ethanol to avoid water coalescence, and potential negative impacts on macromolecule activity due to residual ethanol. [25]

8) Ether infusion technique:
A water-based solution containing the substance is mixed with a lipid solution between 55°C and 65°C in temperature or with a lower pressure. Vacuum evaporation of the ether results in liposomes formation, but this method has drawbacks like producing a heterogeneous liposome population (ranging from 70 to 200 nm) and subjecting the encapsulated materials to elevated temperatures due to the organic solvents. [26, 27]

9) Double emulsification:
By first forming a water-in-oil (W/O) emulsion by using lipid-rich oil and water, then emulsifying in an aqueous phase with surfactants to produce a water-in-oil-in-water (W/O/W) structure, double emulsification produces liposomes that encapsulate hydrophilic substances. [28] Commercial products such as DepoCyte, DepoDur, and Expel are utilized for the production of multivesicular liposomes. [29]

10) Reverse-phase evaporation:

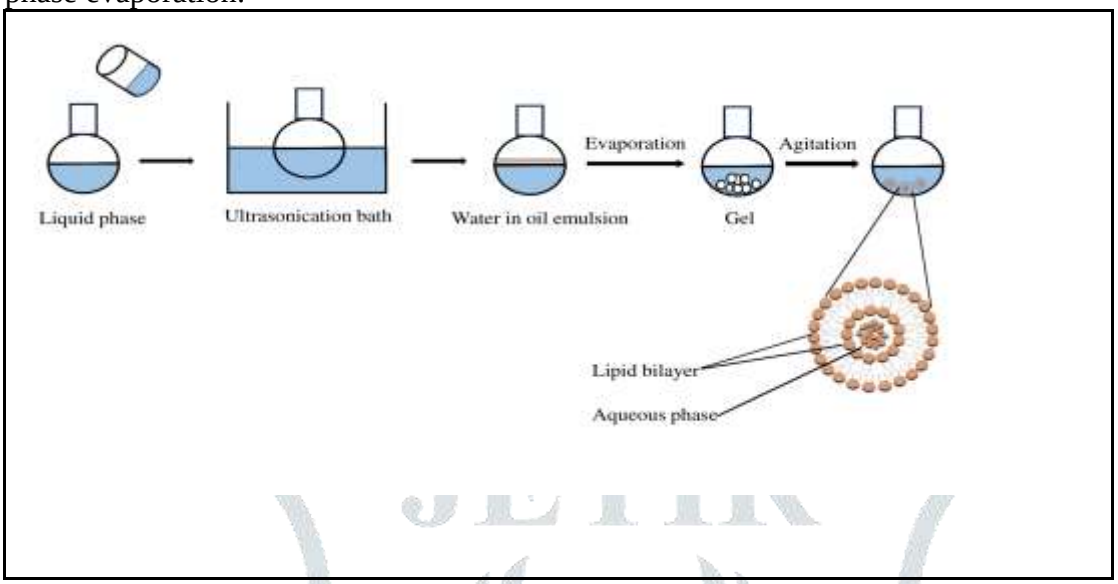

Fig.5: Reverse Phase Evaporation Method

To produce a water-in-oil-in-water (W/O/W) microemulsion, lipids are solubilized in a mixture of chloroform and methanol, combined with an aqueous drug buffer, and subjected to sonication. The creation of liposomes takes place due to the elimination of the organic solvent through reduced pressure. As the solvent evaporates, a viscous gel forms and becomes a semi-transparent liquid, indicating successful liposome formation. Subsequently, a supplementary buffer is incorporated into the liposome suspension and gently vortexed to ensure adequate dispersion. [30]

11) Detergent Solubilization Technique:

The method of detergent removal improves lipid hydration and solubilization through the use of a detergent solution. The detergent engages with phospholipids, safeguarding their hydrophobic regions from water and creating mixed micelles. When the detergent is eliminated, these micelles accumulate additional lipids, causing formation of unilamellar vesicles. Typical examples of detergents include sodium cholate, Triton X-100, sodium deoxycholate, and alkyl glycoside, all of which are noted for their elevated critical micelle concentration (CMC). [18, 31] Detergent removal methods from liposomal solutions include:

1. Dialysis using standard bags or Slide-A-Lyzer.
2. Utilization of organic polystyrene beads (for instance, XAD-2) for the detergent absorption that have minimal micellar concentration. [32]
3. Pretreated beads used in gel permeation effectively capture amphiphilic lipids, allowing the intact liposomes to pass through. [32, 33]

12) Proliposomes:

Proliposomes utilize a soluble carrier to encapsulate both lipids and drugs, resulting in a free-flowing granular substance that generates an isotonic liposomal suspension when hydrated. This method presents a hopeful avenue for the economical mass production of liposomes, especially for lipophilic pharmaceuticals. [22] Proliposome technology features stable phospholipid formulations that generate liposomes when water is introduced prior to application. These vesicles can be further refined into nanosized variants and can also be manufactured on a larger scale through techniques such as high-pressure homogenization. [34]

13) Lyophilization:

Lyophilization is a method that removes water from frozen products under low pressure, ideal for drying heat-sensitive substances. It also addresses long-term stability issues in liposomal formulations, though there is a risk of leakage of encapsulated materials during freeze-drying and reconstitution. [27]

B) Active Loading:

Weakly acidic or basic drug compounds can be incorporated into pre-existing liposomes through active or passive loading methods. This is permitted by an electrochemical potential generated by pH or ion gradients around the membrane consisting of lipids. [35] Active loading techniques are essential to encapsulate hydrophilic drugs in liposomes. [36] They establish a transmembrane gradient that facilitates the influx of drug molecules to enter the

core of liposome, thereby enhancing encapsulation efficiency and at the same time maintaining stability and controlled drug release. The third-generation liposomes enhance the specificity of interactions with sick cells, thereby promoting receptor-mediated endocytosis of both the liposome and its therapeutic contents into the designated cells. [18, 37]

1) PH Gradient Technology: This method modifies acidity and alkalinity in aqueous environments across the lipid bilayer, creating a trans-membrane pH gradient. It utilizes the dissociation changes of weakly acidic or basic drugs at different pH levels, allowing these medications to maintain a low-polarity molecular structure in the surrounding aqueous environment. [38, 39]

2) Ammonium Sulfate Gradient Method:
This technique is particularly suited for weakly basic drugs that exhibit amphoteric characteristics, including Adriamycin, Epirubicin, Daunorubicin, and Mitoxantrone, making it especially beneficial for anticancer medications. The underlying mechanism of this method is somewhat intricate. [39]

3) Calcium Acetate Gradient Technique:
This technique entails the extraction of calcium acetate from the surrounding aqueous phase through methods such as dialysis, ultrafiltration, or ion exchange, which results in generating a concentration gradient. This approach leverages the ionization and hydrolysis of calcium acetate to promote the movement of acetate molecules from the inner aqueous phase of liposomes, where their concentration is elevated, to the outer phase, where the concentration is lower. Consequently, this diffusion process elevates the pH of inner aqueous phase, thereby indirectly establishing a transmembrane pH gradient. [40]

3.2 Novel Methods of Liposome Preparation:
To overcome the stability problems of liposome formulations, new methods have been developed. These are as follows:

1) Freeze Drying Method:
Lipids and active ingredient compound are dissolved in tert-butyl alcohol at a temperature of 45°C, while a lyoprotectant is dissolved in water at the same temperature. Subsequently, these solutions are amalgamated to produce a consistent monophasic solution, which is filtered and freeze-dried to yield proliposomes. During the rehydration process, liposomes with an average diameter of 100 to 300 nm have resulted by freezing at - 40ºC and drying at ambient temperature. [5, 41]

Advantages:
- Appropriate for extensive commercial production.
- Prolongs the durability of liposomes.
- Stops the deterioration of liposomes while in storage.
- Simplified one-step procedure. [5]

Disadvantages:
- The process is labour-intensive and requires significant energy.
- Potential for physical changes, such as variations in vesicle size.
- There is a risk of losing encapsulated substances. [5]

2) Dual Asymmetric Centrifugation Method (DAC):
This innovative centrifugation technique involves rotating sealed vials around both their own axis and the central axis, in contrast to traditional methods that solely rotate around the central axis. [42] This dual rotation generates mechanical turbulence and cavitation, aiding in the formation of uniformly sized nano-liposomes of about 60 nm. [43]

Advantages:
- The equipment is compact and user-friendly
- It offers reproducibility in results.
- The process yields liposomes with a reduced particle size.
- Water-soluble medications exhibit a high entrapment efficiency.
- This approach does not need the organic solvents. [43]

Disadvantages:

- A significant quantity of phospholipids is necessary to achieve adequate viscosity.
- Production is limited to batch scale. [43]

3) Supercritical Fluid Methods (SCF):
Supercritical fluid methods have been introduced as green technology alternatives to prevent the liposomes from becoming toxic and deteriorating. It involves the following methods. [44]

A) Supercritical Anti-solvent [SAS] Technique: This approach employs supercritical carbon dioxide as an antisolvent, requiring it to be fully miscible with the liquid solvent. A phospholipid solution is dissolved in a solvent which is organic, and which is atomized into supercritical carbon dioxide, causing lipid precipitation as fine particles due to reduced solubility. [45] The organic solvent undergoes purification using pure carbon dioxide, and the particles are rehydrated in a water-based buffer to create liposomes. [46]

B) Supercritical CO2 Reverse Phase Evaporation Process: Lipids are incorporated into a batch-mode view cell that is 60 °C hot and filled with supercritical carbon dioxide while being compressed by 10 to 30 bars. After reaching equilibrium, an aqueous glucose solution is added, resulting in large uni-lamellar vesicles with diameters between 200 to 1200 nm. As the concentration of lipids decreases, the dimensions of the liposomes shrink to a range between 100 and 250 nm. [5]

C) Rapid Expansion of Supercritical Solution Technique [RESS]:

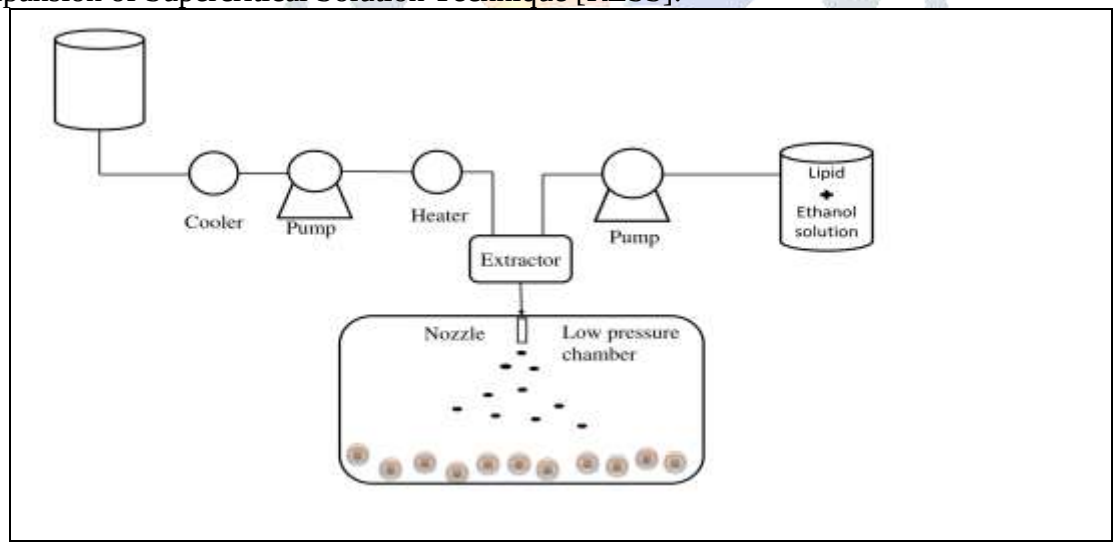

Fig.7: Rapid Expansion of Supercritical Solution Method

Lipids are solubilized in supercritical carbon dioxide with an ethanol concentration of 5 to 10% and then pushed through a narrow opening into a water-based drug solution. The swift reduction in pressure leads to the breakdown of lipids, creating layers that surround the droplets. [47]

D) Expanded Liquid Organic Solution Suspension Depressurization Technique [DELOS]: The approach involves dissolving phospholipids in a solvent under controlled temperature and pressure, then mixing with supercritical CO2 in a cosolvent vessel. Liposomes form when depressurized through a nozzle. A key advantage over the PGSS technique is its compatibility with thermo-sensitive materials, operating at lower temperatures and moderate pressures (10 MPa at 35 °C). [48]

E) Super-Critical Assisted Liposome Formation: In order to create an expanded fluid, lipids are solubilized in ethanol and infused with pure carbon dioxide in a high-pressure saturator. This saturator produces supercritical fluid by heating narrow bands employing baffles. The mixture is atomized at 40 °C and 100 bar in a high-pressure chamber containing an aqueous drug solution. [18] While CO2 and ethanol are separated using a stainless-steel separator at 30°C and 10 bar of pressure, the liposome suspension is gathered from the chamber's bottom. [49] Encapsulation efficiency is influenced by the aqueous solution's flow rate; increased rates result in diminished entrapment efficiency. [50, 51, 52]

Advantages:

- The process is continuous and can be replicated.
- It is utilized for encapsulating hydrophilic drugs.
- It demonstrates high entrapment efficiency and results in minimal solvent residue. [50, 51]

Disadvantages:

- The process is time-consuming and requires the use of high pressure. [50, 51]

4) Solvent-assisted active loading technology (SALT): This method effectively incorporates poorly water-soluble drugs into liposomes by utilizing a minimal quantity of a water-miscible solvent., improving drug solubility and permeation for optimal loading. [16]

4. Evaluation:

Liposomes are evaluated for physical, chemical, and biological properties.

1) Physical characterization
2) Chemical characterization
3) Biological characterization

4.1 Visual appearance:

Liposome suspensions can be either clear or opaque, influenced by the size and concentration of the particles. A uniform grey color may signal non-liposomal dispersions, while turbidity with a bluish tint indicates even particle distribution. An optical microscope is capable of detecting liposomes larger than 0.3 micrometers and can also identify contamination from larger particles. [52]

4.2 Particle size:

The liposome size can be determined by:

a) Optical Microscopy:

The microscopic technique utilizes a Brightfield microscope, a phase-contrast microscope, and a fluorescent microscope. This technique can be used to evaluate the size of large vesicles. [53]

b) Cryo-Transmission Electron Microscopy Technique (cryo-TEM):

This method assesses vesicle size and surface features at −196 °C to preserve sample integrity for natural visualization, avoiding agglomeration and membrane damage typical in traditional TEM while maintaining membrane structure and hydration. [53]

c) Dynamic Light Scattering:

One crucial laser light scattering technique for evaluating liposomes is dynamic light scattering. It measures scattered light intensity to analyze particle size distribution, polydispersity index (PDI), as well as stability, allowing researchers to assess formulation consistency and detect aggregation or size changes over time. [54] Dynamic light scattering effectively assesses the size of liposomes, yielding consistent and uniform distribution results. Gel exclusion chromatography easily determines the hydrodynamic radius. Sepharyl-S100 isolates liposomes from 30 to 300 nanometers. Sepharose-4B and -2B columns differentiate small unilamellar vesicles (SUVs) and micelles. [22]

d) Hydrodynamic Techniques:

The hydrodynamic method is employed to evaluate particle dimensions, membrane fluidity, and the efficiency of encapsulation. [4]

4.3 Zeta potential:

It is a largely utilized for assessing the liposome's surface charge, which may exhibit cationic, anionic, or neutral characteristics, and serves as a fundamental approach for the characterization of liposomes. Surface charge affects the retention and release of encapsulated drugs. [55] High absolute values (± 30 mV or greater) signify strong repulsion and stable dispersion, whereas low absolute values (less than ± 10 mV) suggest increased aggregation and diminished stability. [4]

4.4 Entrapment efficacy:
The fraction of the whole drug which is successfully encapsulated within liposomes is measured by encapsulation efficiency. The amount of the drug enclosed in the liposome relative to the initial amount of the drug to determine this efficiency, and any unencapsulated drug is isolated via ultracentrifugation. The effectiveness of entrapment can be assessed using the subsequent formula:
Entrapment efficiency = (Amount of drug entrapped) / (Total amount of drug) × 100. [56]

4.5 Trapped volume:
The trapped volume in liposome evaluation refers to the amount of aqueous solution within the liposomal vesicles, which is crucial for assessing drug delivery efficacy as it affects the retention of the active ingredient.
1) Size Exclusion Chromatography:
This technique differentiates free molecules from those in liposomes, but liposome retention in the gel matrix may affect accuracy. A study investigates liposome retention in size exclusion chromatography (SEC) and its potential impact on lipid depletion and sample contamination. [57]
2) Fluorescence or UV Spectroscopy: Fluorescence techniques are non-invasive methods used across various fields. The emergence of compact solid-state UV emitters has significantly enhanced the adoption of UV fluorescence techniques, which activate a variety of fluorophores using ultraviolet light. [58] In UV Spectroscopy, the absorbance of a liposome-encapsulated compound is measured at a specific wavelength. Changes in absorbance before and after liposome disruption quantify the encapsulated volume. [59]

4.6 Drug release:
In vitro dispersion of aligned cells enables detailed analysis of drug release from liposomes, improving pharmacokinetic and bioavailability predictions and minimizing costly in vivo studies. A method was developed to evaluate drug release from liposomes using mouse liver lysosomal lysate, assessing pharmacokinetic efficacy through drug bioavailability and other release metrics into plasma and buffer post-degradation. [29]

4.7 Liposome stability:
Ideal liposomes are stable physically, chemically, and physiologically. Physical stability depends on the drug-to-lipid ratio and size uniformity, while chemical stability is affected by oxidative and hydrolytic degradation, especially in unsaturated fatty acyl chains. To avoid oxidation, liposomes should be shielded from light and oxygen and stored at low temperatures. Sterilized cationic liposomes can maintain stability at 4 °C for extended periods. [52]

4.8 Surface charge:
Liposome surface properties, including charge, are affected by head group composition, influencing kinetics, distribution, and cell interactions. Surface charge is measured using free-flow electrophoresis of multilamellar vesicles (MLVs) on a cellulose ester plate in sodium borate buffer at PH 8.8, with samples electrophoresed at 4°C for 30 minutes to separate liposomes by charge. [5]

4.9 Anticancer Activity Evaluation:
This study assesses a liposome's anticancer effects on cell lines using MTT, Trypan blue exclusion, and CCK-8 assays, as well as apoptosis analysis via Annexin V staining as well as Caspase activity assays.
1) MTT assay: The MTT test is a colorimetric method commonly for assessing cellular metabolic activity. It involves the utilization of NAD(P)H-dependent oxidoreductase enzymes in living cells to transform MTT dye into an insoluble purple formazan compound. In addition to the electron acceptor PMS, other tetrazolium dyes such as XTT, MTS, and WSTs, are also utilized. WST-1, which is unable to penetrate cellular membranes, is diminished externally via the electron transport mechanism of the plasma membrane. [60]

2) Trypan blue exclusion assay**:** This assay measures cytotoxicity by counting viable cells in suspension with a dye like trypan blue, which stains only dead cells. [61]

3) Cell Counting Kit-8 assay: This approach evaluates the cytotoxic impact of liposomes on cells by determining cell viability through the amount of colored formazan dye produced. [62, 63]

4) Annexin V-staining: This method evaluates the apoptotic effects of liposome-formulated anti-cancer agents by detecting phosphatidylserine (PS) on cell membranes, with positive Annexin V staining indicating apoptosis. [64, 65]

5) Caspase Activity Assay: The procedure includes incubating liposomes with cancer cells and measuring caspase activity to assess the liposomes' ability to induce apoptosis. [66, 67]

4.10 Particle Size Determination:

A particle sizer is used to measure the mean diameter and polydispersity index (PDI) of liposomes. The particle size uniformity is measured by the PDI. A low PDI (<0.3) indicates stable liposome dispersion, while a high PDI (>0.5) suggests instability and requires optimization. [4]

5. Therapeutic applications of liposomes:

1) Liposome serves as a delivery mechanism, encapsulating various molecules and being compatible with biological systems. Research has examined their pharmaceutical applications, like sustained release, enhanced bioavailability, improved absorption, targeted delivery, and increased vaccine adjuvanticity. [68]

2) In recombinant DNA technology, liposomes are vital for gene therapy, improving gene delivery and aiding gene function research. Their achievement relies on the effective integration of nucleic acids into cells, both in laboratory environments and within living organisms. [69]

3) Liposomes serve as versatile adjuvant carriers in vaccine delivery due to their excellent biocompatibility. [70]

4) Enhance drug solubilization: Liposomes enhance not only the solubility but also the absorption of drugs with low solubility in water by encapsulating hydrophobic substances and ensuring compatibility with biological systems. [71]

5) In Enzyme replacement therapy, encapsulating enzymes enhances their therapeutic effectiveness. Liposomes serve as effective carriers for delivering these enzymes, with their properties adjustable based on membrane composition, size, and fabrication techniques. [72]

6) In tumor and cancer treatment, liposomes deliver chemotherapy directly to tumors, minimizing healthy tissue absorption and side effects while enhancing drug concentration in tumors. [73]

7) In Chelation therapy, Chelation therapy eliminates harmful metals using chelators bind to metal ions. Oral EDTA has low bioavailability, with only 5% absorbed, while intravenous delivery has limited cellular uptake and is quickly eliminated by the kidneys, exhibiting a half-life ranging from 1.4 to 3 hours. [74]

8) One significant barrier to the effective transport of drugs to the brain is the blood-brain barrier (BBB). Liposomes have ability to penetrate the blood brain barrier. [75]

9) In respiratory drug delivery, liposomes are gaining attention in respiratory drug delivery for protecting drugs, improving bioavailability, and targeting lung areas. They are effective for asthma, COPD, lung cancer, and gene therapy, and may help prevent respiratory syncytial virus infections. [76, 77]

10) To deliver food substances: Liposomes transport nutrients such as vitamins, minerals, bioactive compounds, polyphenols, and carotenoids. [78]

11) Liposomes are applicable in the treatment of antimicrobial, antifungal, and antiviral conditions. [79]

6. Challenges in the Development of Liposomes:

1) Immunogenicity: Liposomes can trigger an immune response, leading to inflammation and reduced efficacy. This can be mitigated by using biocompatible lipids and surface modifications. [80]

2) Clinical Translation: Despite encouraging preclinical results, the adoption of liposomal drug delivery systems in clinical settings has been gradual, primarily due to the need for extensive trials, regulatory challenges, and the complexities of creating disease-specific liposomes. [81]

7. Future Prospective:

Researchers are developing new methods for improving drug efficacy, reducing need of multiple dosing, and increasing drug loading in liposomes.

8. Conclusion:

Liposomes have found extensive application across various areas of pharmaceuticals. Furthermore, therapeutic medicines can be precisely delivered to the affected cells because of liposomes. Liposomal formulations are recognized for their low toxicity and improved therapeutic effectiveness. However, there are certain limitations associated with the formulation of liposomes.

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