

Nanoliposome: An overview of types, preparation, evaluation and application

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Abstract

This review article intends to provide an overview of liposomes and nanoliposomes their types, classification and properties, methods of preparation, patented technology for the formulation of nanoliposomes. Characterization and evaluation parameters of nanoliposomes. Also it explains various applications of nanoliposomes in nanotherapy including in food industry, antimicrobial delivery, diagnostics, and cosmetics, improving pharmacological properties, rheumatoid Arthritis, chemotherapy, Alzheimer Disease, gene therapy, and nutraceuticals. Nanoliposomes are a new concept, whether liposomes carry a past of more than five decades. First time Bangham *et al* were described liposomes in 1961. The word liposome originates from two Greek words, 'lipo' means fat and 'soma' means body or structure that means a structure in which aqueous compartment(s) are encapsulated into a fatty shroud.

Although the concept of liposome was known from five decades ago, current studies claimed the presence of these lipid vesicles in our very first natural meal, i.e., breast milk. Nanoliposomes were described by Mozafari and Mortazavi in 2005. Nanoliposome is a nano carrier having one or more semi core lipids formed by water and lipid sources present between two lipid layers. Nanoliposomes are the vesicles containing concentric bilayers of phospholipids with both hydrophobic and hydrophilic regions. Hydrophilic drugs are entrapped in the aqueous section and hydrophobic and amphiphilic drugs are found position in concentric bilayer of phospholipids. Drugs encapsulated in these systems may be distributed to the targeted site, provided that the stability of bonded carrier molecules with the drug during the passage from the administration site to the targeted site. This advantage, together with biocompatibility and biodegradability, make nanoliposomes very fascinating as "smart" drug delivery vehicles.

Keywords: Liposome, Nanoliposome, Nanocarriers, Nanotherapy, Biomedical applications.

Introduction

Discovery of Liposomes and Nanoliposomes

Nanoliposomes are a new concept, whether liposomes carry a past of more than five decades. First time Bangham *et al* were described liposomes in 1961 [Bangham and Horne, 1964]. The concept of liposome was known from five decades ago, current studies claimed the presence of these lipid vesicles in our very first natural meal, i.e., breast milk.

The word liposome originates from two Greek words, 'lipo' means fat and 'soma' means body or structure that means a structure in which aqueous compartment(s) are encapsulated into a fatty shroud. Alec Bangham first described how exclusive structures of liposome were formed by interactions of phospholipids with water [Bangham 1978].

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He observed that on combination with water phospholipids immediately transformed into a sphere like structure because each molecule carry one water soluble and other water insoluble end. Water soluble medications were entrapped into the aqueous core while the lipophilic medications were integrated into the phospholipids layer. Nano carriers improved the natural distribution of drug and decreased side effects allied with the drug by regulating the rate of release of drug and improving the solubility profile of drug [Dass and Choong, 2006].

Nanoliposomes

Nanoliposomes whereas described by Mozafari and Mortazavi in 2005. Nanoliposome is a nano carrier having one or more semi core lipids formed by water and lipid sources present between two lipid layers [Latif and Bachhawat, 1984; Peer *et al.*, 2007]. Nanoliposomes are the vesicles containing concentric bilayers of phospholipids with both hydrophobic and hydrophilic regions. Hydrophilic drugs are entrapped in the aqueous section and hydrophobic and amphiphilic drugs are found position in concentric bilayer of phospholipids [Riaz, 1996]. Drugs encapsulated in these systems may be distributed to the targeted site, provided that the stability of bonded carrier molecules with the drug during the passage from the administration site to the targeted site.

As carriers, nanoliposomes protect the active ingredient from the surrounding dispersion medium against chemical degradation [Garcia-Fuentes *et al.*, 2005] and control the rate of release of incorporated compound. Nanoliposomes can maintain their nanostructure integrity and drug cross the nanoliposomal membrane through diffusion and then the BBB of malignant gliomas through the pores. Nanocarriers are preferably used via systemic route due to their ability of prolonged circulation in the bloodstream. However, after intravenous administration, they interrelate with the reticuloendothelial system (RES), and removed rapidly from systemic circulation [Moghimi *et al.*, 2001]. Depending on their size, charge, and surface properties [Ogawara *et al.*, 2001]. To minimize the rapid plasma clearance, the nanocarriers are coated with chitosan or poly (ethylene glycol) (PEG) or directly link with

either chitosan or PEG chemically [Laquintana *et al.*, 2009].

The surfaces of nanocarriers are modified with polymers to prepare a hydrated shield around the particle to modify the stability of nanocarriers in a slightly alkaline environment [Moreno *et al.*, 2010]. The coating delays the clearance of nanoliposomes. Mononuclear phagocyte system cleared the uncoated nanoliposomes rapidly from liver, spleen and bone marrow depending on their size, surface modification, and solubility profile. Different coating materials can be used to modify the solubility and to increase the stability of nanocarriers depending on the need.

Chitosan is the most widely used coating materials provide a shielding effect to the nanoliposomes by escalating the charge on the surface of particles. The character of the coating material plays an important role in reducing the aggregation and improving the steadiness of nanocarriers within the body. Surface of nanoliposomes can be modified by coatings with biocompatible, hydrophilic, and biodegradable polymers like chitosan. It is a naturally occurring alkaline, biocompatible, biodegradable, and hydrophilic polymer with no toxicity. Chitosan has wide application in gene delivery. Chitosan improved the permeation of particles to target the drug at desired site like ocular [De Campos *et al.*, 2001], nasal [Fernandez *et al.*, 1999; Vila *et al.*, 2004], buccal [Portero *et al.*, 2002], intestinal [Artursson *et al.*, 1994; Borchard *et al.*, 1996; Prego *et al.*, 2005], and pulmonary [AlQadi *et al.*, 2012].

Nanoliposomes has referred to as nanoscale bilayer lipid vesicles since the term liposome is definition, including various types of vesicles with average size up to several micrometers (Mozafari and Mortazavi, 2005; Patil and Jadhav, 2014). Nanoliposomes present a greater surface area and have acceptable stability profile to preserve their size with in nanometric scales, e.g., as small as 20–100 nm (small liposomes) and >100 nm (large liposomes) (Khorasani *et al.*, 2018). These carriers are mainly composed of lipids and phospholipids. However, some contain molecules, such as carbohydrates, proteins, antioxidants, sterols in their structure (Mozafari and Khosravi-Darani, 2007). Due to their amphiphilic nature, they have the potential to entrap and release a massive range

of hydrophilic and hydrophobic compounds simultaneously providing a combined benefit. Additionally, their characteristic bilayer structure is highly compatible with the skin surface, allowing them to act as penetration enhancers of

bioactive compounds toward targeted sites (Farghaly *et al.*, 2017). This advantage, together with biocompatibility and biodegradability, make nanoliposomes very fascinating as “smart” drug delivery vehicles.

Table 1. Vesicle types with their size and number of lipid layers.

Vesicle Types	Abbrev	Diameter Size	Number of lipid bilayers
Unilamellar vesicles	UV	All size range	One
Small unilamellar vesicles	SUV	20-100 nm.	One
Large unilamellar vesicles	LUV	> 100nm.	One
Giant unilamellar vesicles	GUV	> 1µm.	One
Multilamellar vesicles	MLV	> 0.5µm.	Five to twenty
Oligolamellar vesicles	OLV	0.1-1µm.	Approximately five
Multivesicular vesicles	MMV	> 1µm.	Multi compartmental structure

CLASSIFICATION OF LIPOSOMES ON THE BASIS OF METHOD OF PREPARATION

Table 2. Different preparation methods and the vesicles formed by these methods.

Preparation method	Vesicle types
Single or oligo lamellar vesicle made by reverse phase evaporation method	REV
Multi lamellar vesicle made by reverse phase evaporation method	MLV-REV
Stable pluri lamellar vesicle	SPLV
Frozen and thawed multi lamellar vesicle	FAT MLV
Vesicle prepared by extrusion technique	VET
Dehydration – Rehydration method	DRV

METHODS OF PREPARATION OF NANOLIPOSOME

The conventional methods for preparing nanoliposomes include solubilizing the lipids in organic solvent, drying down the lipids from organic solution, dispersion of lipids in aqueous media, purification of resultant liposomes and analysis of the final product. Some of the commonly used methods for the preparation of nanoliposomes are described below.

Mechanical dispersion method

The following are types of mechanical dispersion methods:

- a) Sonication.

- b) French pressure cell: extrusion.
- c) Micro- fluidizer
- d) Lipid Layer Hydration Method
- e) Emulsification-Evaporation Method
- f) Heating Method
- g) Reverse-Phase Evaporation Method
- h) Mozafari Method
- i) High Pressure Homogenization Method
- j) Modified Emulsification and Ultrasonic Method

Solvent dispersion methods

- a) Ether Injection Method
- b) Ethanol Injection Method

Detergent removal method (removal of non - encapsulated material)

- a) Dialysis

b) Detergent removal of mixed micelles

Sonication method

Sonication is a simple method for reducing the size of liposomes and manufacture of nanoliposomes (Jesorka, Orwar, 2008). This method is based on size transformation and involves the subsequent sonication of MLVs. Transfer the MLVs either to a probe sonicator or a bath sonicator. For probe sonication, place the tip of the sonicator in the MLV flask and sonicate the sample with 20 s ON, 20 s OFF intervals, for a total period of 10–15 min (Prabhu *et al.*, 2010). At this stage, nanoliposomes are formed, which are mostly in the form of small unilamellar vesicles (SUV). Otherwise, nanoliposomes can be produced using a bath sonicator.

Probe sonication: The tip of a sonicator is directly immersed into the liposome dispersion. The energy input into lipid dispersion is very high. The coupling of energy at the tip results in local hotness; as a result, the vessel must be deep into a water/ice bath. All through the sonication up to 1 h, more than 5% of the lipids can be de-esterified. Also, with the probe sonicator, titanium will pollute the solution.

Bath sonication: The liposome dispersion in a cylinder is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method, in compare to sonication by dispersal directly using the tip. The sonicated material can be protected in a sterile vessel under an inert atmosphere.

Sonication is a trouble-free technique for plummeting the magnitude of liposomes and for the preparation of nanoliposomes (Figure 3) [Mozafari, 2010; Woodbury-Smith *et al.*, 2006; Jesorka and Orwar, 2008; Narsaiah *et al.*, 2012]. In this method, hydrated lipid vesicles are sonicated with probe sonicator in an ice bath for several minutes. The phospholipid components and cholesterol are mixed in chloroform-methanol by sonication and the mixture is dried by lyophilization or spray drying [Szoka and Papahadjopoulos, 1980]. Then, the dried lipids are hydrated with a fluid containing chelating agents, salts, cryo-protectants, stabilizers, and the drug to be encapsulated. At first, micrometric type liposomes will be formed which are sonicated for a required time period [Arab Tehrani *et al.* 2012]. Prepared chitosan-coated nanosize liposomes by

sonication method and studied the different factors that has been affected the loading efficiency and payload of vitamin C by using high-pressure liquid chromatography [Liu and Park, 2010]. Narsaiah *et al.*, prepared nanoliposomes loaded with pediocin and hybrid alginate to study the effect of various process parameters on nanoliposomes size like quantity of phospholipids, amplitude for sonication and sonication time, and to compare the release profile of pediocin after loaded into different nanoformulations that were prepared using different materials [Narsaiah *et al.*, 2012].

French pressure cell (extrusion)

Extrusion is a process in which involves the conversion of micrometric liposomes (e.g. MLV) are structurally modified to large unilamellar vesicles (LUV) or nanoliposomes depending on the basis of filters of different pore-size used (Hope *et al.*, 1985; MacDonald *et al.*, 1991; Berger *et al.*, 2001; Zucker *et al.*, 2011). Vesicles are physically extruded under pressure through polycarbonate filters of defined pore sizes. This procedure has advantages over the sonication method. In this method a variety of membrane pore sizes are available for producing liposomes in different selected size ranges, and in addition, the size distribution of the liposomes can be made fairly narrow, mainly by cycling the material through the selected-size filter several times. The membrane extrusion method has several drawbacks in large-scale processing. For one, the pores in the membrane tend to clog up, predominantly when processing concentrated suspensions and/or when the liposome sizes are substantially greater than the membrane pore sizes. The clogged membranes cannot be cleared and replacing the filter is likely to compromise the sterility of the extrusion operation. Secondly, the membranes themselves are planar disks which must be mounted against a flat mechanical support. This harshly restricts the surface area available for extrusion, and leads to deliberate throughput. Although the problems of clogging and deliberate throughput can be overcome partially at high extrusion pressures. The flow diagram of Extrusion technique is shown in (Figure 4). Nanoliposomes can also be prepared via extrusion. Lipids are dissolved in chloroform in their respective molar ratios. A nitrogen stream

is passed to evaporate the organic solvent and to obtain a dried lipid film which is hydrated with phosphate-buffered saline containing drug by using a vortex mixer. Unilamellar liposomes are immediately sonicated at room temperature [Liu and Yang, 2012].

Micro-fluidization/ Micro-emulsion

This is a method of nanoliposome production without using potentially toxic solvents. The microfluidization technique using equipment called microfluidizer. It is based on the principle of dividing a pressure stream into two parts, passing each part through a fine orifice, and directing the flows at each other inside the chamber of microfluidizer (Jafari *et al.*, 2006). Within the interaction chamber, cavitation, along with shear and impact, reduces particle sizes of the liposomes. Microfluidizer uses high pressures (up to 10,000 psi) to direct the flow stream through microchannels toward the impingement area (Sorgi *et al.*, 1994). The reduction in the size can be achieved by recycling of the sample. The process is reproducible and yield liposomes with high-quality aqueous phase encapsulation. Microfluidizer is used to formulate small vesicles from concentrated lipid concentration. The lipid can be introduced into the fluidizer as a suspension of large MLVs. The first MLVs were prepared by these were passed through a Microfluidizer at 40 psi inlet air pressure. The size varied from 150-160 nm after 25 recycles.

Lipid Layer Hydration Method

This method involves conversion of lipid into a thin film by evaporating the organic solvent and then by using different aqueous solvents hydrating this film. Lipid layer hydration method can also be used to prepare nanoliposomes (Figure 5) [Markoutsas *et al.*, 2012; Pedrosa *et al.*, 2013]. A desired amount of lecithin, cholesterol, and butyrate hydroxyl anisole is liquefied in chloroform and mixed vigorously manually. The mixture is then rotated in a vacuum evaporator to evaporate the solvent. Then, the deionized water with drug is used to hydrate the lipid film. The dispersion is sonicated and the preparation is reserved at room temperature for vesicle formation and finally the preparation is stored at low temperature overnight. Mozafari *et al.*, used the heating method and the thin-film hydration

method to prepare anionic nanoliposomes [Mozafari *et al.*, 2007].

Guan *et al.*, prepared nanoliposomes by using three methods like reverse-phase evaporation method [Szoka and Papahadjopoulos, 1978], thin film hydration method [Guan *et al.*, 2011], and ether injection method [Deamer and Bangham, 1976], to select the best method for the preparation of lactoferrin nanoliposomes and to examine their steadiness under different conditions, particularly in the pretend gastrointestinal tract [Guan *et al.*, 2011]. Further, they also compared the three different methods of preparation of the lactoferrin nanoliposomes based on their size distribution and encapsulation efficiency and to estimate the uptake of lactoferrin nanoliposomes by cells and their stability [Guan *et al.*, 2012]. Rasti *et al.*, prepared nanoliposomes by thin-film hydration method and Mozafari method and compared the steadiness of recently prepared and stored liposomal and nanoliposomal systems containing docosahexaenoic acid and eicosapentaenoic acid on the basis of their oxidation [Rasti *et al.*, 2012]. Pedrosa *et al.*, used lipid film hydration and extrusion method to prepare nanoliposomes with short chain spingolipids to improve the delivery of doxorubicin in tumor cells [Pedrosa *et al.*, 2013]. Markoutsas *et al.*, prepared mono- and dual-decorated nanoliposomes by immobilization of MAb against transferrin and/or a peptide analogue of ApoE3 to target low-density lipoprotein receptor (Re *et al.*, 2010; Markoutsas *et al.*, 2014).

Emulsification-Evaporation Method

Nanoliposomes can also be prepared by using the emulsification-evaporation and low temperature curing method. The schematic representation of the emulsification-evaporation method is shown in (Figure 6). The temperature of aqueous phase is maintained at 75°C. The oil phase composition is melted and retained at 80°C. Drug and phospholipids are then added in the oil phase and then injected into the aqueous phase through plastic needle tubing under mechanical agitation. After stirring, the resulting suspension is kept at a low temperature. The nanoliposomes are obtained by filtering the resulting suspension through a filter membrane of 0.2 µm to remove the non-incorporated drug [Wang *et al.*, 2012]. Prepared

nanoliposomes by emulsification- evaporation method and studied the hybrid molecular-nanoliposomal biocompatible compositions *in vitro* effect on cultured peritoneal cells [Shkurupiy *et al.*, 2008].

Heating Method

An appropriate mixture of the phospholipids is hydrated with an aqueous medium containing cholesterol in presence of nitrogen or argon. The hydration of nanoliposomal ingredients depends on their solubility and phase transition temperature. The lipid dispersions and the content to be entrapped are mixed in a heat-proof flask with 3% glycerol by placing it on a hot-plate stirrer, above the phase transition temperature. Mozafari and Mortazavi prepared anionic nanoliposomes by the heating method and the thin-film hydration method [Mozafari and Mortazavi, 2005]. It is used three methods to prepare PEG-coated oxaliplatin loaded nanoliposomes like thin film hydration method, reverse-phase evaporation method, and heating method to investigate the influence of temperature, presence of cholesterol and the use of different cryoprotectants in lyophilization on their stability [Zalba *et al.*, 2012]. It is used heating method to prepare flavourzyme nanoliposomes without employing any chemical solvent or detergent [Jahadi *et al.*, 2015].

Reverse-Phase Evaporation Method

Szoka and Papahadjopoulos, originally introduced this method to prepare nanoliposomes [Mozafari *et al.*, 2002]. The schematic representation of the emulsification-evaporation method is shown in (Figure 7). A suitable amount of phospholipid components and cholesterol is dispersed in organic solvent like diethyl ether, chloroform, and drug is added in a phosphate-buffer. The probe sonication is used to mix the organic phase with the aqueous phase. The organic solvent is evaporated under reduced pressure till the mixture is converted into a gel by using a rotary evaporator. Then, desired amount of phosphate-buffer solution containing Tween 80 is added and sonicated for another 20 min [Fan *et al.*, 2007; Liang *et al.*, 2008; Zalba *et al.*, 2012]. Prepared lactoferrin nanoliposomes by reverse-phase evaporation method, thin film hydration method, ether injection method to opt the best method to prepare lactoferrin nanoliposomes and

to investigate their stability under different conditions [Guan *et al.*, 2011]. It is developed chelating ligand-bound nanoliposomes for the prevention and reversal of β -Amyloid aggregation associated with promoting neurotoxicity in Alzheimer disease [Mufamadi *et al.*, 2012].

Mozafari Method

Mozafari and Mortazavi, 2005 prepared nanoliposomes without any filtration or sonication depending on the style of flask used and its number of baffles, its kind, speed and period of mixing, ingredients of nanoliposomes, sample volume [Mozafari and Mortazavi, 2005; Colas *et al.*, 2007]. Rasti *et al.*, prepared nanoliposomes by thin-film hydration method and Mozafari method and compared the stability of freshly prepared and stored liposomal and nanoliposomal preparations of docosahexaenoic acid and eicosapentaenoic acid [Rasti *et al.*, 2012].

High Pressure Homogenization Method

Coarse lipid suspension is prepared by homomixer and their size is reduced to nanorange by using high pressure homogenizer. Kang *et al* prepared nanoliposomes by using unsaturated lecithin and studied various factors like solvents, pH balance, homogenizing pressure, co- surfactants and stabilizers to prepare stable nanoliposomes [Kang *et al.*, 2005]. Prepared, characterized and evaluated the safranalnanoliposomes as a natural sunscreen and moisturizer factor [Golmohammadzadeh *et al.*, 2011]. Alexander *et al.*, prepared plant sterols loaded nanoliposomes by using high pressure homogenization method to examine the consequence of phytosterols on the encapsulation efficiency and stability of soy phospholipids vesicles [Alexander *et al.*, 2012]. Prepared tea polyphenol extract loaded nanoliposomes by using milk phospholipids with high-pressure homogenization method to sustain their rate of release and to study their influence on the adenocarcinoma cell line viability [Gulseren *et al.*, 2012].

Modified Emulsification and Ultrasonic Method

Modified emulsification and ultrasonic method was also used to formulate nanoliposomes (Figure 1.6) [Guan *et al.*, 2011]. Drug was liquefying in anhydrous ethanol to attain a desired concentration of drug ethanolic solution. The hydrophobic phase comprised of phospholipids, cholesterol, and ethanolic solution of drug was warmed on a

water bath at 60 Tween 80 was added in 10ml of phosphate buffer of pH 6.8 and maintained at same temperature as the hydrophobic phase. Soya lecithin and cholesterol were mixed completely in ethanolic solution of drug. Under magnetic stirring the aqueous phase was dropped into the hydrophobic phase continuously when soya lecithin and cholesterol was completely mixed with ethanolic solution of drug. The consequential preparation was stirred for another 10 min, and then ultrasonication was performed using probe sonicator for 10 min with a 3s pulse-on and a 1s pulse-off period. Then, the preparation placed on an ice bath and diluted to a desired volume. Finally the preparation was filtered through a 0.22µm membrane filter.

Solvent dispersion methods:

Ethanol Injection Method: The lipid solution of ethanol is quickly injected to a excess amount of buffer and the MLVs are immediately formed. The major drawbacks of the method are the production of heterogeneous products (30-110 nm) and liposomes are very dilute. It is difficult to remove the entire ethanol because it forms azeotrope with water and the possibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol (Batzri and Korn, 1973).

Ether Infusion Method: The lipids dissolved in diethylether or ether/methanol mixture is slowly injected to an aqueous solution of the material to be encapsulated at 55-65°C or under

reduced pressure. The subsequent removal of ether under vacuum leads to the formation of liposomes. The major drawbacks of the method are the production of heterogeneous (70-190 nm) products and the exposure of compounds to be encapsulated to organic solvents or high temperature (Loxley., 2009).

Detergent removal method (removal of non - encapsulated material):

Dialysis Method

The detergents at their critical micelle concentrations (CMC) have been used to solubilize lipids. As the detergent is detached, the micelles become increasingly better-off in phospholipid and lastly combine to form LUVs. The detergents were removed by dialysis. The dialysis can be performed in dialysis bags deep in large detergent free buffers.

Detergent removal of mixed micelles

The absorption of detergent is attained by shaking mixed micelle solution with beaded organic polystyrene absorbers such as XAD-2 beads, and Bio- beads SM2. The main benefit of using detergent absorbers is that they can eliminate detergents with a very low CMC, which are not entirely exhausted.

Patented Methods of Formulation of Nanoliposomes

Various methods for the preparation of nanoliposomes have been patented. Some of the patented techniques are enlisted in Table 3. Some of the patents are:

Table 3: Some Examples of Patented Technologies for the Formulation of Nanoliposomes

Type of Nanoliposomes	Patent #	Method of formulation	References
Nanoliposome using esterified lecithin containing epidermal growth factor	US 20090263473 A1	Sonication method	Dadgar <i>et al.</i> , 2014
Heavy Metal-Containing Nanoliposomes	US 20100112040 A1	Lipid layer hydration method, High pressure homogenization method, Sonication method, Microfluidization method, Extrusion method	Hong <i>et al.</i> , 2009
Nanoliposomes for alopecia treatment	WO 2010027217 A2	Sonication method	Basheer, 2010
Cholecalciferol nanoliposomes	WO 2010090502 A2	Sonication method	Hong <i>et al.</i> , 2010
Flexible nanoliposomes	CN 102793665 A	Film dispersion method	Antimisiari <i>et al.</i> , 2012
Nanoliposomes of medium chain fatty acids	CN 101940321 B	High-pressure-microfluidization, freezing and	Weizheng <i>et al.</i> , 2012

		thawing method	
Macromolecular-phosphatidylglycerol nanoliposome	CN 103565744 A	Membrane dispersionmethod	Lee <i>et al.</i> , 2013
Teaphenol nanoliposomes	CN 103637989 A	Ethanol injection- dynamic high-pressure microfluidization-enzymolysis method	Guirong and Party, 2014

CHARACTERIZATION AND EVALUATION OF NANOLIPOSOMES

The different preparation of nanoliposomes, particularly when using a new technique, characterization is required to make sure adequate quality of the product. Each and every technique has characteristic advantages and probable disadvantages. The most important parameters of nanoliposome characterization include visual appearance, size distribution, stability, Zeta potential, lamellarity and entrapment efficiency.

Visualization

Visualization can be observed by an optical microscope (phase contrast) that can detect particles size greater than 300 nm and contamination with larger particles. The size distribution of nanoliposomes is mainly determined using electron microscopy. The more newly developed microscopic technique with very high resolutions is the Scanning Probe Microscopy (SPM). One of the most applied SPM techniques are Scanning Tunnelling Microscopy (STM) and Atomic Force Microscopy (AFM). This new technology gives the possibility to view various biological and non-biological samples under air or water with a resolution up to 3Å (Ozer., 2007).

Size Determination

The electron microscopy and the light scattering technique are used to determine the size distribution of nanoliposomes. The light scattering technique helps in measuring the size of different vesicles in an aqueous medium [Markoutsa *et al.*, 2012]. For a comprehensive and reliable characterization of nanoliposomal formulations, these techniques may be engaged with other economical and regular laboratory techniques [Narsaiah *et al.*, 2012; Mozafari *et al.*, 2007; Mortazavi *et al.*, 2005]. Light scattering provides collective information about the size of nanoliposomes. It presumes every aggregation of various vesicles as a particle. On the other hand,

electron microscopic techniques provide information about the shape and aggregation or fusion of the vesicles.

Zeta Potential

Zeta potential is the overall charge in a lipid vesicle acquires in a particular medium. It is a measure of the magnitude of repulsion or attraction between particles in general and lipid vesicles in particular. Evaluation of the zeta potential of a nanoliposome preparation can help to predict the stability and in vivo outcome of liposomes [khosravi-Darani *et al.*, 2007]. Understanding of the zeta potential is also useful in controlling the aggregation, fusion and precipitation of nanoliposomes, which are important factors affecting the stability of nanoliposomal formulations [Mozafari *et al.*, 2005].

Lamellarity Determination

Lamellarity of vesicles is the number of bilayer present in liposomes which are determined by using Freeze- Fracture Electron Microscopy and P-31 Nuclear Magnetic Resonance (31426 P NMR) analysis (Jesorka, Orwar., 2008). Additional techniques for lamellarity determination comprise electron microscopy, small angle X-ray scattering (SAXS), and also the methods that are based on the change in the visible or fluorescence upon the addition of reagents.

Encapsulation Efficiency

The most common method to determine the encapsulation efficiency of nanoliposomes is to encapsulate a hydrophilic marker like fluorescent dye, and radioactive ion and different techniques like spectrophotometry, enzyme based methods, fluorescence spectroscopy, and electrochemical techniques can be used depending on the nature of the encapsulated material [Jesorka and Orwar, 2008; Chatterjee and Banerjee, 2002; Edwards and Baeumner, 2006]. To estimate about the leakage of content from nanoliposomes and to

check the effect of different disrupting conditions on encapsulated material any purification technique can also be used [Ding *et al.*, 2011].

In Vitro Release

Drug release from nanoliposomal formulation is estimated by using the dialysis bag method [Meng *et al.*, 2011]. A suitable volume of preparation is put in a dialysis bag. Dialysis tubing consisted of regenerated cellulose and it's both ends are tied. The dialysis bag is suspended in media in which *in vitro* release of drug was performed and maintained the media at 37 ± 0.5 °C. The dispersion is shaken by using a magnetic bead at specified rpm for uniform distribution of contents. 1 ml aliquots are removed and replaced with 1 ml fresh phosphate buffer solution, at suitable interval of time to maintain the uniform volume of phosphate buffer solution. The temperatures of samples are also maintained. Drug concentrations are quantified using HPLC [Dadgar *et al.*, 2014].

Entrapment Efficiency

The entrapment efficiencies of prepared nanoliposomes were determined by finding the concentration of free drug in the dispersion medium. The obtained suspension was centrifuged for 60 min at 10,000 rpm. The supernatant was separated and then filtered through 0.45 µm Millipore. The filtrate was diluted using 75% ethanol and measured spectrophotometrically. The amount of free drug was determined in the filtrate and the amount of incorporated drug was determined as a result of the initial drug minus the free drug. The entrapment efficiency was calculated using the following equation.

$$\text{Entrapment efficiency} = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100$$

Where "W initial drug" is the mass of initial drug used and the "W free drug" is the mass of free drug

measured in the supernatant after centrifugation of the aqueous dispersion.

Percentage yield:

The percentage yield is calculated to know about the efficiency of any method, thus it helps in selection of appropriate method of production. Practical yield was calculated as the weight of nanoparticles recovered from each batch in relation to the sum of starting material. The

percentage yield of prepared nanoliposomes was determined by using the formula.

Formula for Percent Yield

$$\text{Percent yield} = \frac{\text{Actual Yield}}{\text{Theoretical Yield}} \times 100\%$$

Applications of Nanoliposomes

Nanoliposomes are used in various fields (Figure 1.7) starting from drug and gene delivery to diagnostics, food nanotechnology, cosmetics, and long-lasting immunocontraception [Gabizon *et al.*, 1994]. In aqueous media, the self-sealing properties of nanoliposomes are responsible for their various applications in different fields like pharmaceuticals, food and cosmetics [Narsaiah *et al.*, 2012; Chaudhry *et al.*, 2008].

In Food Industry: The main application of nanoliposomes is varying the texture of food components, regulating the discharge of flavors, encapsulating the food components or additives, revolutionize the new flavor and sensations, and escalating the bioavailability of nutritional components [Thompson, 2003; Kirby, 1991]. In dairy industry nanoliposomes are used to encapsulate the food preservatives. An example is the preservation of washed curd cheeses, such as Gouda, Emmental, and Edam [Allena and Cullis, 2013].

In Antimicrobial Delivery: Nanoliposomes have been used for targeted delivery of antimicrobial agents at the desired sites [Bergers, 1995]. Nanoliposomes and liposomes are used as potential carriers for the delivery of bactericides to treat the infectious diseases [Gabizon, 1994; Colas, 2007; Moribe and Maruyama, 2002; Foldvari, 1996]. Nisin-loaded nanoliposomes are prepared without the use of any toxic solvents and harmful procedure [Gabizon, 1994; Colas, 2007]. Nanoencapsulated pediocin is more effective bacterial growth inhibitor than free pediocin [Narsaiah *et al.*, 2012]. The nanoliposomal form of minocycline hydrochloride showed a strong inhibitory effect for extended time period on macrophagic cell tumor necrosis factor- α (TNF- α) secretion, which is stimulated by lipopolysaccharide (LPS) than minocycline hydrochloride solution and pericloine [Liu and Yang, 2012].

In Cosmetics: Nanoliposomes have been widely used in sunscreen formulations. They demonstrate distinctive features like easy drug delivery, excellent reservoirs for drug, stabilizing the drug, and water resistance properties. Various factors influence drug deposition into the skin layers which includes the physicochemical properties of the drug and other ingredients, size and lipid composition of particles, charge, vehicle, lamellarity of particles and method of application of formulation [103-106]. Nanoliposomes prepared by using esterified lecithin are used to prepare cosmetics having excellent moisturizing and penetrating properties in skin, and containing medicament for treating skin diseases. This composition entrapped epidermal growth factor in nanoliposomes, which stimulated the skin-penetration and increase the pharmaceutical stability [Hong *et al.*, 2009]. Oleanolic acid is entrapped in nanoliposomes as an active ingredient to increase the percutaneous absorption and to encourage the synthesis of collagen, ultimately showing a good anti-wrinkle effect [Muller *et al.*, 1996].

In Improving Pharmacological Properties: Nanoliposomes improved the pharmacological properties of drugs. Also, they protect the biological activity of drugs, control the release of drug, and improve the stability and efficacy by extending half-life [Dimitrov, 2006]. Nanoliposomes can be easily absorbed by cells because they can easily pass through the body's smallest capillaries and blood brain barrier due to their small size [Schnyder and Huwyler, 2005; Liu *et al.*, 2006; Gharib *et al.*, 2012]. The pharmacological efficacy of Ticarcillin-loaded cationic nanoliposomes is increased many times against *P. aeruginosa* infection than free, anionic

and neutral ticarcillin formulations [Avnir *et al.*, 2011].

In Rheumatoid Arthritis: Rheumatoid arthritis is an autoimmune disease of the joints which can be concealed by glucocorticoids with or without other drugs. Glucocorticoids are quickly cleared from the circulation, and thus, adversely affect other tissues. It has been reported that nanoliposomes loaded with methylprednisolone hemisuccinate or with betamethasone hemisuccinate have shown excellent therapeutic efficacy in an adjuvant arthritis model of rheumatoid arthritis in Lewis rats when injected i.v. [Avnir *et al.*, 2008; Wang *et al.*, 2011].

In Chemotherapy: Magnetic nanoliposomes have various activities like thermotherapy with magnetic nanoparticles, gene therapy with various modified complexes and chemotherapy using As₂O₃ [Zucker *et al.*, 2010]. The nanoliposomes improved the therapeutic efficacy of a combination of topotecan (TPT) and vincristine (VCR). The co-encapsulation of these synergistic anticancer drugs manipulated the therapeutic results and decreased the side effects of chemotherapy [Zucker *et al.*, 2010]. Microbubble-nanoliposome complex has been used as diagnostic and therapeutic agents for breast, colon, pancreatic, oesophageal, liver, stomach, lung, prostate, ovarian and kidney cancer by cancer-cell-specific diagnosis and treatment [Chulhak and Yihakcheol, 2014]. Basheer, 2010 filed a patent for

Heavy metal- containing nanoliposomes which are used to treat certain disorders like inflammatory conditions, cancer, and metal deficiency-related diseases [Basheer, 2010]. Some of the marketed preparations containing nanoliposomes are enlisted in Table 4.

Table 4: Some Examples of Marketed Preparations Containing Anticancer Drugs in formof Nanoliposomes

Drug Name	Active Ingredient	Company	Reference
Camptosar or CPT-11	Irinotecan Hydrochloride	Pfizer, New York, NY	Friedman <i>et al.</i> , 1999; Gilbert <i>et al.</i> , 2003; Chou <i>et al.</i> , 2005
DaunoXome®	Daunorubicin	Nexstar Pharmaceuticals	Allen and Martin, 2004; Immordino <i>et al.</i> , 2006

DOXIL®/Caelyx®	Doxorubicin	Sequus Pharmaceuticals	Immordino <i>et al.</i> , 2006
Myocet®/Evacet®	Doxorubicin	Elan Pharma, U.S.A	Alberts <i>et al.</i> , 2004; Immordino <i>et al.</i> , 2006
Lipodox®	Doxorubicin	Sunpharma, India	Immordino <i>et al.</i> , 2006

In Alzheimer Disease (AD): In Western countries, AD is the fourth broad reason of death subsequent to cancer, heart disease, and stroke. It is the common form of dementia occurring mostly in elder population with neurodegenerative diseases [Brookmeyer *et al.*, 2002]. The structural distinctiveness essential for the antifibrillogenic activity retained by the curcumin decorated nanoliposomes. They demonstrated the highest affinity for amyloid- β 1-42 fibrils and are very valuable for diagnostic and therapeutic purposes by targeting these AD pathogenic markers [Mourtas *et al.*, 2011].

Conclusion

Nanoliposome, or submicron bilayer lipid vesicle, is a new technology for the encapsulation and delivery of bioactive agents. The list of bioactive material that can be incorporated to nanoliposomes is huge, ranging from pharmaceuticals to cosmetics and nutraceuticals. nanoliposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life and reducing toxicity. The sole characteristic of nanoliposomes is their ability to compartmentalize and solubilize both lipophilic and hydrophilic surroundings in one system, and thus, they are appropriate for the delivery of all categories of drugs like amphipathic, hydrophobic, and hydrophilic drugs, coupled with biocompatibility and biodegradability make nanoliposomes very attractive as drug delivery vehicles. nanoliposomes provide more surface area, increase solubility of active components, increase bioavailability of drugs, enable exactitude targeting of the encapsulated material to a huge extent due to their nano size. Over all nanoliposomal formulations are still important and successful accesses for the clinical application of nanomedicines. Therefore, it is sensible to project that this field will experience steady growth for the future prospective.

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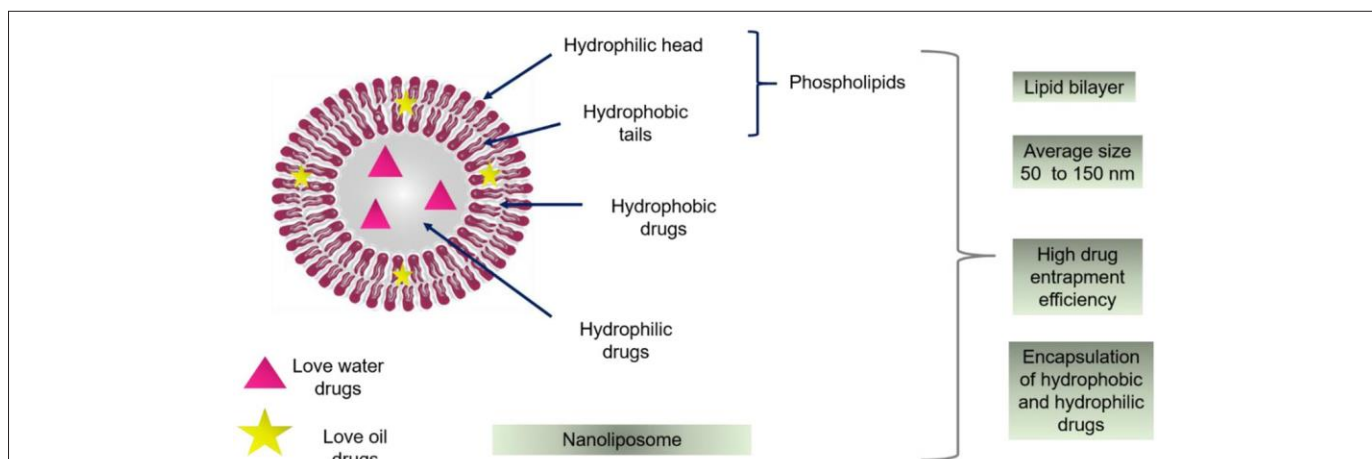


FIGURE 1 | Schematic diagram of the bilayer structure of nanoliposomes with the representation of their amphiphilic structure for the entrapment of hydrophobic and hydrophilic drugs. On the right, some of the remarkable characteristics of these systems are listed. The liposomal structure is mainly composed of phospholipids such as phosphatidylcholine, phosphatidylserine, or phosphatidylethanolamine. Nonetheless, the addition of cholesterol in the liposomal formulation is very common with the purpose of providing stability and

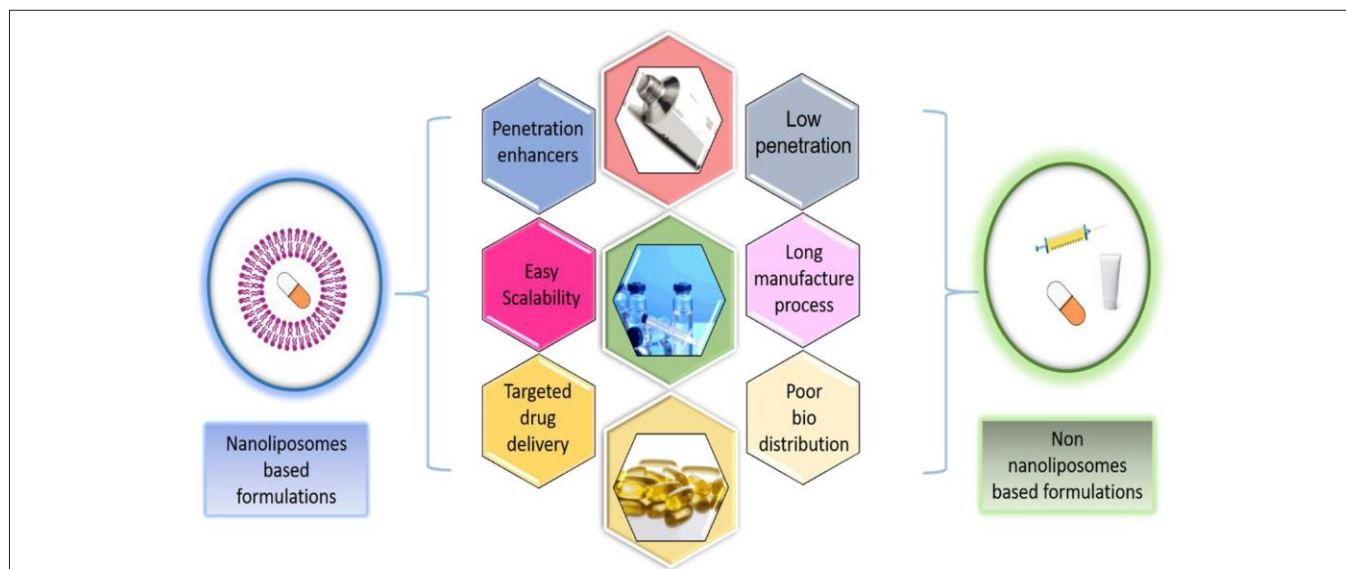


FIGURE 2 | Advantages of nanoliposomes based formulations respect to non-nanoliposomes based formulations for oral, topical, and intramuscular drug administration.

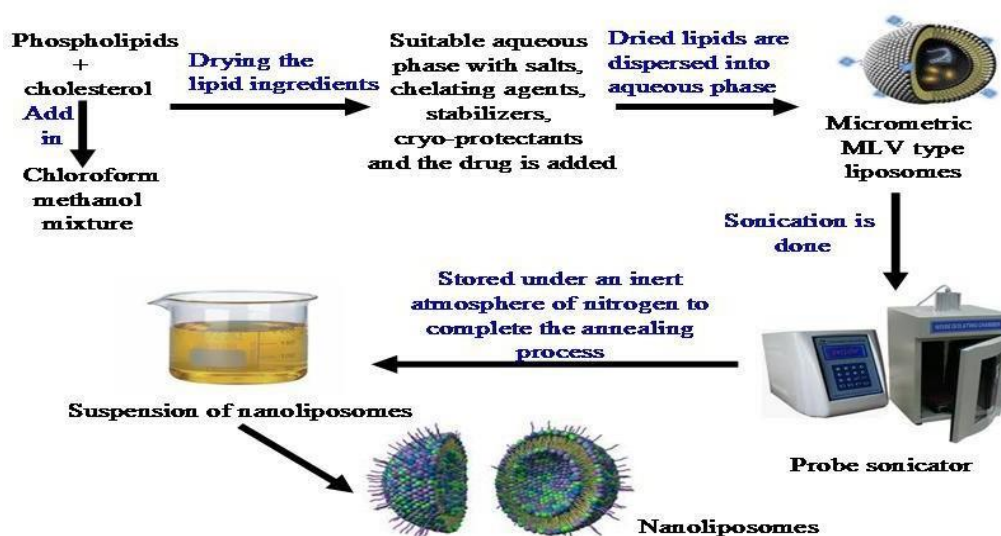


Figure 3: Schematic Representation of the Sonication Technique [Rohilla and Dureja, 2015]

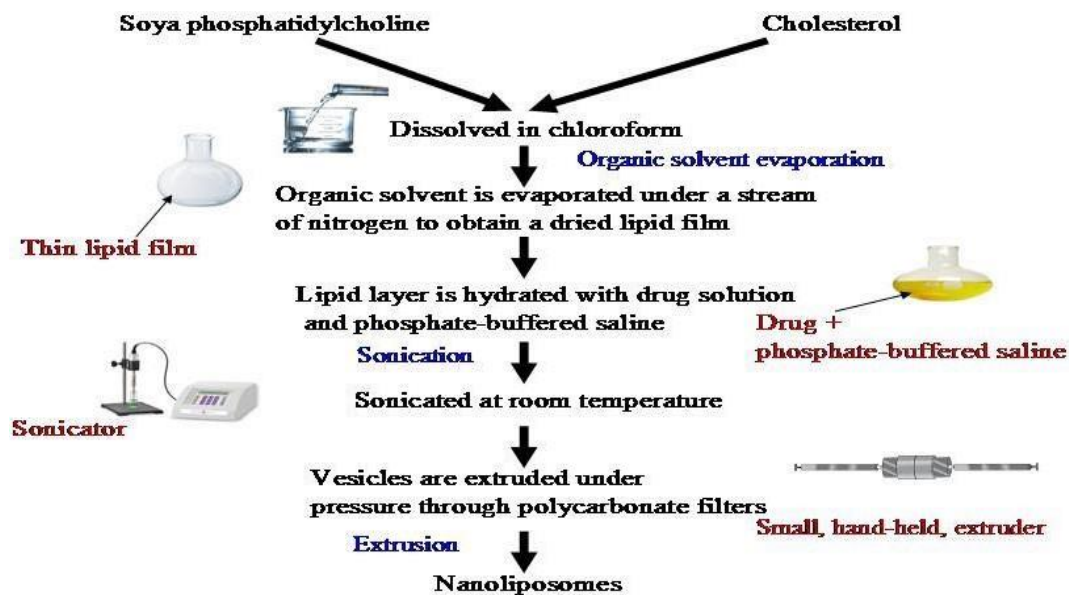


Figure 4: Schematic Representation of the Extrusion Method [Rohilla and Dureja, 2015]

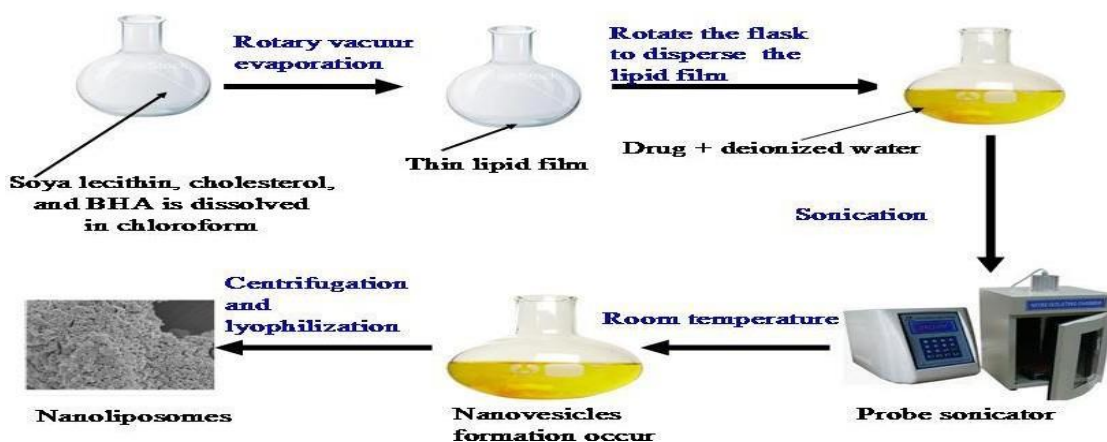


Figure 5: Schematic Representation of the Lipid Layer Hydration Method [Rohilla and Dureja, 2015]

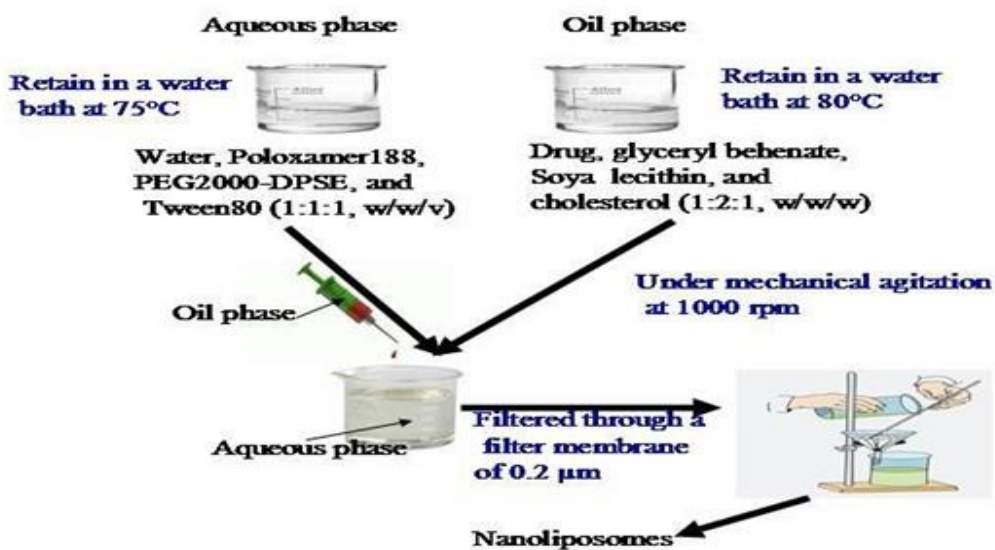


Figure 6: Schematic Representation of the Emulsification-Evaporation Method [Rohilla and Dureja, 2015]

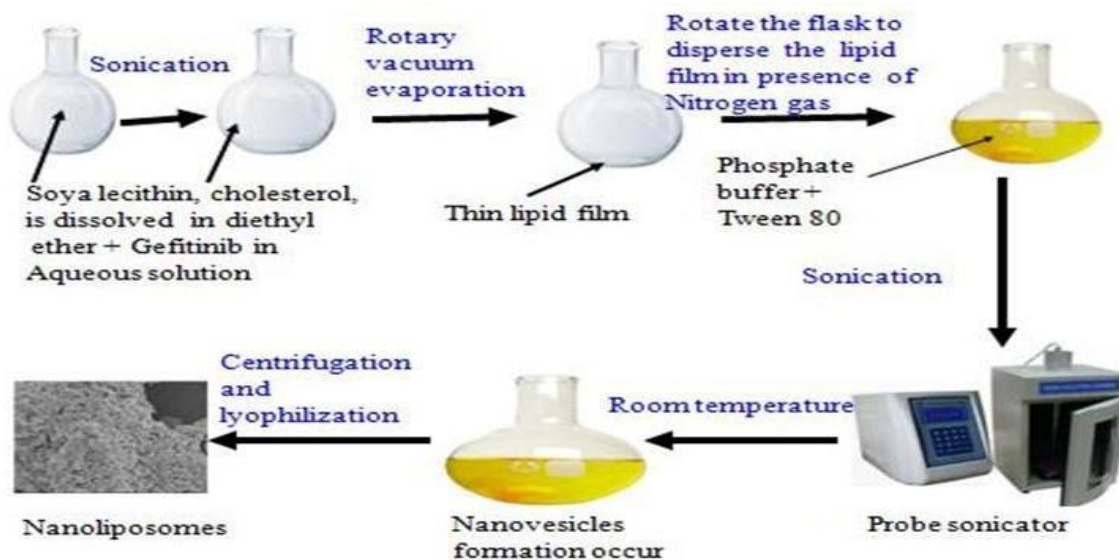


Figure 7: Schematic Representation of Reverse Phase Evaporation Method

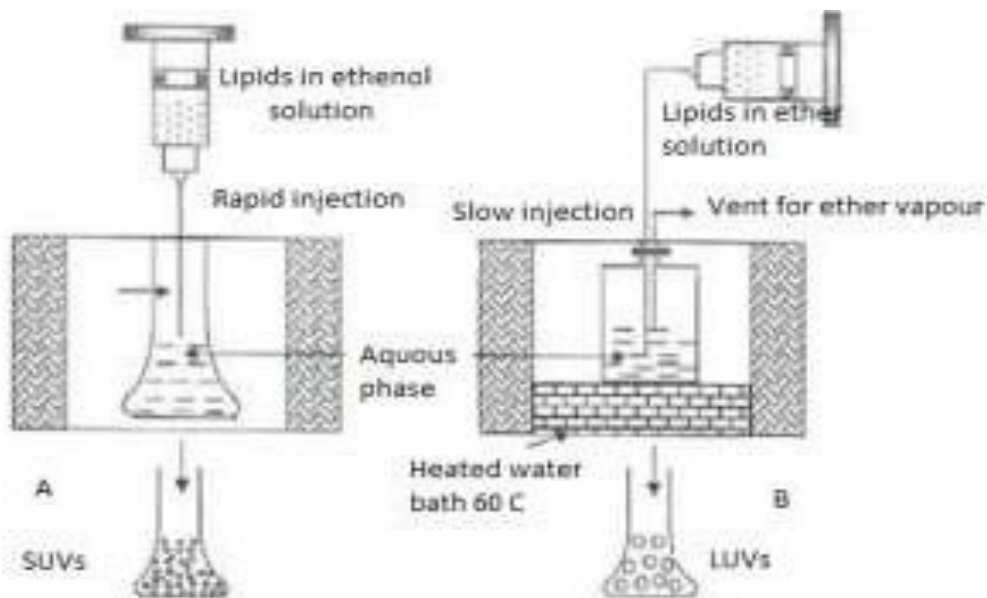


Figure 8. Sonication apparatus for Ethenol injection (A) & Ether injection (B)

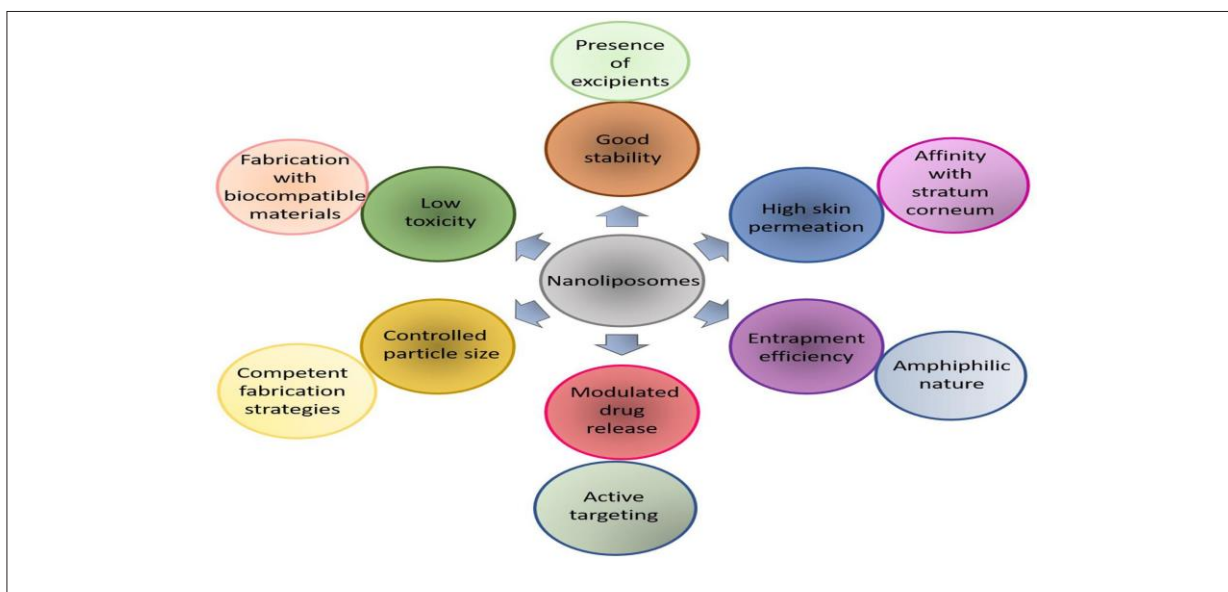


Figure 9: Schematic representation of multi-functional characteristics of drug loading into nanoliposomes as a competent model for biomedical applications.

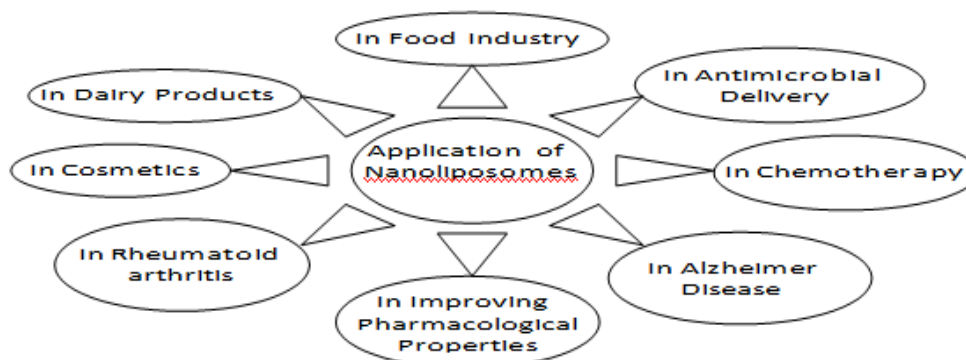


Figure 10: Schematic Representation of Application of Nanoliposomes

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