

# Stability Aspects of Liposomes

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## ABSTRACT

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Stability testing is the primary tool used to assess expiration dating and storage conditions for pharmaceutical products. Many protocols have been used for stability testing, but most in the industry are now standardizing on the recommendations of the International Conference on Harmonization (ICH). These guidelines were developed as a cooperative effort between regulatory agencies and industry officials from Europe, Japan, and United States. Proper design, implementation, monitoring and evaluation of the studies are crucial for obtaining useful and accurate stability data. Stability studies are linked to the establishment and assurance of safety, quality and efficacy of the drug product from early phase development through the lifecycle of the drug product. Liposomes have been extensively investigated for drug delivery, drug targeting, controlled release and enhancing solubility. However the major limitation in the widespread use of this versatile drug delivery system is its instability. In present review the methods like control of the particle size, lipid composition, method of drug loading, prodrug, pro-liposomes, lyophilization and electrosteric stabilization are reviewed to enhance the physical, chemical and biological stabilities of the liposomes collectively because all are inter-related.

**Keywords:** Liposomes, Stability, Steric, Lyophilization.

## INTRODUCTION

Liposomes or 'lipid bodies' sometimes called as "vesicles" are structures formed spontaneously by polar lipid molecules or amphiphilic molecules, each having polar head group and long hydrophobic tail. e.g. phospholipids such as lecithin's. Structurally liposomes comprise of an outer shell of one or more membrane-like bilayers of the molecule arranged concentrically around hollow interior of "vacuole" which can serve as storage compartment for the active agent. In the outer layer the polar heads of the molecules are oriented outwardly of the liposomes while hydrophobic tail e.g. palmitic acid, stearic acid inwardly. If there are multiple layers the orientation is reversed in the alternate layer so that the lipid tails of one layer intermingle with lipid tails of the next, and the polar heads of one layer about those of a neighbor. Active agent stored in the interior of liposomes, where surrounding membrane amphiphilic molecules shelters them, may or may not be dissolved in aqueous media, and are typically hydrophilic. The liposomes are dispersed in an aqueous medium or polar solvent medium. Lipophilic active agent can also be carried by liposomes locating themselves in the lipid

layers formed by the hydrophobic tails of the amphiphilic structured liposomal material. Gas filled liposomes are also known. Liposomes have been extensively investigated for drug delivery<sup>1,2,3</sup>, drugs targeting<sup>4,5,6,7,8</sup>, controlled release<sup>9</sup> and increased solubility<sup>10</sup>. The major rate-limiting step in the use of this versatile drug delivery is the stability both physical and chemical. If liposomes are developed to enter market as products, they must be stable during the storage period, and remain at the appropriate size and intact before reaching their targeted tissues and producing action. Considerable attention has been given to study the stability of liposomes for last three decades, but there remains much work to be done because the instability problems still limit their application. The present paper intends to review classification of stability of liposomes, methods of enhancement of physical, chemical, biological stabilities collectively because all are inter-related.

## CLASSIFICATION OF STABILITY OF LIPOSOMES

Liposome stability can be subdivided into physical, chemical and biological stabilities, which are all inter-related. Generally, the shelf-life stability of liposomes is determined by the physical and chemical stability (Uniformity of size distribution and encapsulation efficiency, and minimal degradation of all compounds, respectively). By optimizing the size distribution, pH and ionic strength, as well as the addition of antioxidants and chelating agents, liquid liposome

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formulations can be stable. As phospholipids usually form the backbone of the bilayer their chemical stability is important. Two types of chemical degradation reactions can affect the performance of phospholipid bilayers: hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and peroxidation of unsaturated acyl chains (if present). The oxidation and hydrolysis of lipids may lead to the appearance of short-chain lipids and then soluble derivatives will form in the membrane, resulting in the decrease of the quality of liposome products<sup>11</sup>. Moreover, physical processes such as aggregation/flocculation and fusion/coalescence that affect the shelf life of liposomes can result in loss of liposome-associated drug and changes in size. Aggregation is the formation of larger units of liposomal material; these units are still composed of individual liposomes. In principle, this process is reversible<sup>12</sup> e.g. by applying mild shears forces, by changing the temperature or by binding metal ions that initially induced aggregation. However, the presence of aggregation can accelerate the process of coalescence of liposomes, which indicates that new colloidal structures are formed<sup>12</sup>. As coalescence is an irreversible process; the original liposomes cannot be retrieved. A colloidal dispersion is often thermodynamically unstable. Spontaneous processes occur in the direction of decreasing Gibbs free energy; therefore the separation of a two-phase dispersed system to form two distinct layers is a change in the direction of decreasing Gibbs free energy<sup>13</sup>. There is more surface energy in a liposome suspension when the dispersed phase is in a highly subdivided state than when it is in a coarser state of subdivision. The central feature of coalescence is the fact that the total surface area is reduced in the coarsening process of thermodynamically unstable liposome dispersion, while there is no reduction of surface in aggregation, although certain surface sites may be blocked at the points at which the smaller particles touch<sup>13</sup>. After small particles coalescence, only the new larger particle remains. With aggregation, however, the small particles retain their identity and the aggregation moves as a single unit.

Biological stability of liposomes, however, depends on the presence of agents such as proteins that interact with liposomes upon application to the subject and depends on the administration route. Strategies used to enhance biological stability of liposomes will improve liposome-mediated drug delivery in vivo and increase circulation time in the blood stream<sup>14</sup>. It was observed that aerosols of interleukin 2 liposomes were biologically stable and retained in the lung after nebulization<sup>15</sup>. Incorporating steric stability, e.g. the incorporation of N-acyl-phosphatidyl ethanolamine into liposomes has shown to increase the liposomal biological stability towards plasma components<sup>15</sup>.

## FUNDAMENTALS OF COLLOID STABILITY

### DLVO theory<sup>16</sup>:

Although no general model exists for the colloid stability of liposomes, the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory can quantitatively explain the physical stability of liposomal systems. The total interaction potential ( $V_{\text{tot}}$ ) between approaching particles is a function of the repulsive component ( $V_{\text{R}}$ ) of the interaction potential and the attractive component ( $V_{\text{A}}$ )

$$V_{\text{tot}} = V_{\text{R}} + V_{\text{A}} \quad (1)$$

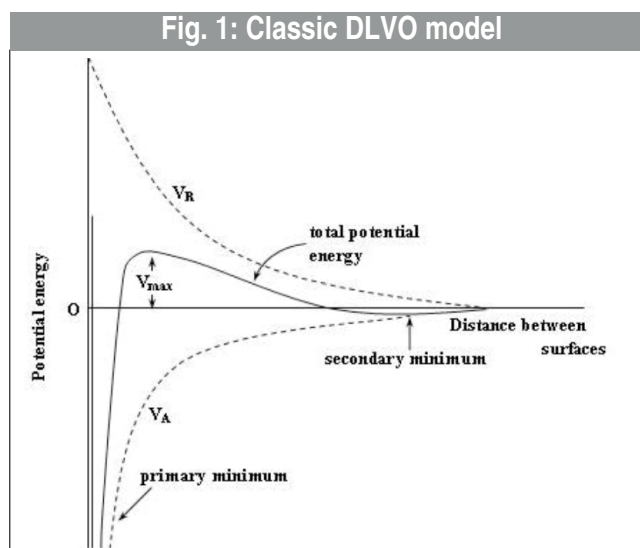
$$V_{\text{R}} = 64 \pi a n k_{\text{B}} T \gamma^2 \exp(-\kappa H) / \kappa^2 \quad (2)$$

$$V_{\text{A}} = -A/6 \{ 2 a^2 / (H^2 + 4 a H) + 2 a^2 / (H + 2a)^2 + \ln(H^2 + 4 a H) / (H + 2a)^2 \} \quad (3)$$

where  $a$  is the particle radius,  $H$  is the surface to surface distance,  $n$  is the number of ions per unit volume,  $\kappa$  is the Debye reciprocal length,  $A$  is the Hamaker constant,  $kT$  is the thermal energy and factor  $\gamma$  is related to the surface potential  $\psi_0$ , electronic charge and ion valency number  $v$ , through

$$\gamma = \tanh(v e \psi_0 / 4 k B T) \approx v e \psi_0 / 4 k B T \text{ (when } \psi_0 \rightarrow 0) \quad (4)$$

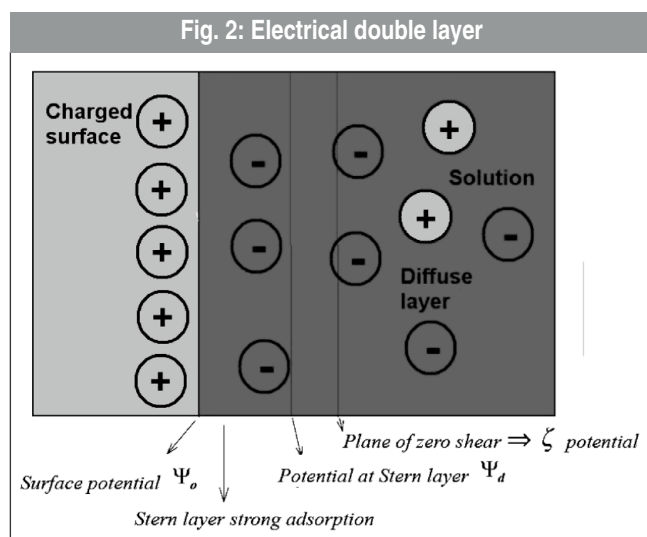
The ubiquitous attractive force between liposomes is Vander Waals attraction, while the long range repulsive force is electrostatic repulsion. The balance between these two factors determines the colloid stability. The electrical forces increase exponentially as particles approach one another and the attractive forces increase as an inverse power of separation. As a consequence, these additive forces may be expressed as a potential energy versus separation curve. A positive resultant corresponds to an energy barrier and repulsion, while a negative resultant corresponds to attraction and hence aggregation. (Fig 1) States that the classic DLVO model is



applicable for determining the stability of liposomes, considering that many of the physicochemical properties of liposomes resemble that of conventional colloidal particles.

**The electrical double layer<sup>17</sup>:**

The foundation of DLVO theory was the postulate of additivity of the dispersion and electrostatic double layer interactions. The later were calculated as pair interactions in an infinite electrolyte reservoir using the Poisson equation with the ion density distribution characterized in terms of the Boltzmannian statistics. In this respect, the DLVO theory can be seen as one of many applications of the Gouy-Chapman-Stern electric double layer model. When a solid surface contacts an aqueous solution, it acquires a charge due to one of the three mechanisms: ionization of surface groups, specific adsorption of ions from solution and the dissolution of ions from the surface. Given that electro-neutrality must be maintained within the system, ions present within the bulk of the aqueous solution move towards or away from the charged surface to form what is called the electrical double layer, which was first put forward in the 1850's by Helmholtz. Within the layer (Fig. 2), the surface charge is balanced by the presence of counter ions. For the charge stabilized colloidal suspensions, the aggregation process is governed by the repulsive energy barrier in the interaction potential between two approaching liposomes. If the height of this barrier is reduced to less than the thermal energy  $k_bT$ , every collision will result in the liposomes sticking together and the system undergoes a very rapid aggregation, limited only by the rate of diffusion-induced collisions between the clusters. If the energy barrier is of the order or larger than  $k_bT$ , many collisions must occur before two liposomes can stick to one another and the system experiences a much slow aggregation. Both types of growth kinetics result in a highly polydisperse cluster mass distribution.



**KINETICS OF THE LIPOSOME AGGREGATION AND COALESCENCE**

**Smoluchowski theory:**

The general treatment for the coagulation kinetics in colloidal systems was performed by von Smoluchowski (1916; 1917). If  $N_0$  is the number of primary vesicles present at  $t = 0$ , then for a low concentration of liposomes in solutions, the total number of vesicles at time  $t$ ,  $N$  can be described as:

$$N = N_0 / (1 + kN_0t) \tag{5}$$

or

$$1/N = 1/N_0 + kt$$

Where  $k$  is the second-order aggregation rate constant.  $N$  may be approximated by

$$N = 6\phi / \pi d^3 \tag{6}$$

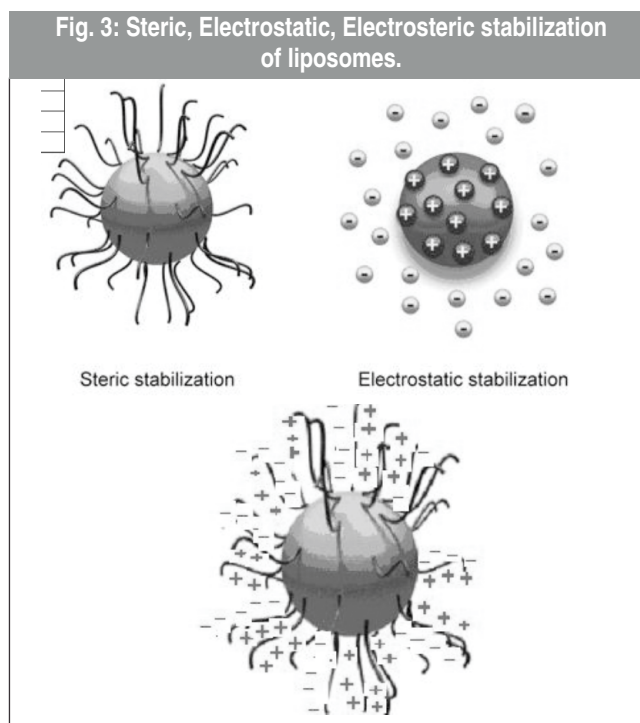
Where  $d$  is vesicle diameter and  $\phi$  is the liposome volume fraction. For liposome systems, the entrapped aqueous volume is difficult to determine and  $\phi$  is hard to be estimated.

Even though Smoluchowski's treatment neglects surface forces and hydrodynamic interactions, the aggregation process in the presence of these interactions remains a second-order process.

From Eqs 5 and 6

$$d_t = [d + (6k\phi/\pi)t]^{1/3} \tag{7}$$

Where  $d_0$  is the liposome diameter at the time  $t$ . By plotting  $d^3$  versus time, we can calculate the rate constant for aggregation,  $k = 6k\phi/\pi$ .



## METHODS FOR ENHANCEMENT OF STABILITY

Maximum encapsulation efficiency and absence of drug leakage i.e. drug retention are the major goals in vesicular drug delivery system. Achieving such goals is possible only if the physical, chemical biological or in vitro / in vivo drug retention stability of liposomes is enhanced.

Drug retention stability depends on the number of factors like, method of manufacture, method of drug loading, lipid composition, physicochemical properties of drug, In present review we tried to explain methods collectively because these are all inter-related

### *Control of particle size and lamellarity:*

Any change in particle size can affect targeting, RES uptake, safety and efficacy. Liposomes are classified according to the size and lamellarity. Aggregation and fusion is mostly observed with particle size < 20 nm that have excessive high stress curvature which promotes this phenomenon. Fusion can be prevented by good manufacturing procedure<sup>18</sup>. Small unilamellar vesicles (SUV), 25 to 100 nm in size that consists of a single lipid bilayer. Large unilamellar vesicles (LUV), 100 to 400 nm in size that consists of a single lipid bilayer. Multilamellar vesicles (MLV), 200 nm to several microns that consist of two or more concentric bilayers. Vesicles above 1

µm are known as giant vesicles. Particle size and lamellarity depends on the method of manufacture it is described in literature<sup>19</sup>. Liposomes can be prepared by several methods as mentioned in the table no. 1

The lamellarity of liposomes influences to a great extent the encapsulation efficiency, the efflux rate of liposomal encapsulated material, and the fate of a drug after cellular uptake<sup>20</sup>. In one study it was observed that liposomes prepared by the combinations of some lipids on storage at 4 and 25°C over 6-month period large unilamellar vesicles (REV) proved to be superior to multilamellar liposomes (MLV) and dehydration/rehydration liposomes (DRV) systems as far as physical stability was concerned. Instability was exaggerated in the systems stored at 25°C as compared to storage at 4°C<sup>21</sup>.

### *Lipid composition:*

Permeability and stability of liposomes are influenced by the rigidity/stiffness of the lipid bilayer. Selection of lipid in turn depends on the phase transition temperature of lipids, which depends on the acyl chain length. Gel-liquid phase transition occurs in a narrow temperature range for pure lipids (T<sub>c</sub>). T<sub>c</sub> is affected by fatty acid side chains, degree of unsaturation, chain length and polar head groups. Lipids with long acyl chain length are most commonly used because high phase transition temperature<sup>19</sup>. All these things are not possible by

Table 1: Preparation methods for liposomes<sup>19</sup>

Classification of methods	Sub-classification of methods	Liposomes obtained
Mechanical dispersion	Hand-shaken method	LUV or MLV
	Pro-liposomes	MLV
	Freeze-drying method	MLV
Physical-hydration ( Modify or Improve Characteristics of liposomes)	Micro-emulsification	MLV
	Sonication (cuphorn, bath or probe tip sonicator)	SUV
	French press extrusion	SUV
	Membrane extrusion	LUV
	Dried-reconstitute	LUV or MLV
	Freeze-thawing sonication (FTS)	SUV or LUV
	Dehydration-rehydration vesicle	SUV
	pH-induced vesiculation	SUV or LUV
	Calcium-induced fusion	LUV
Solvent dispersion	Ether injection	SUV or LUV
	Double emulsification	LUV
	Multiple emulsification	SUV or LUV
	Ethanol injection	SUV or LUV
	Reverse-phase evaporation (RE)	LUV
Other methods	Detergent removal/dialysis	SUV or LUV
	Fusion of SUV	LUV
	Film-ultrasonic technique	Dependable on drug
	Amphiphiles-loading	Dependable on drug

using single lipid; this can be achieved by combinations of lipids or incorporation of another substances. Temperature affects phosphatidylcholine (PC) liposomes stability and the optical densities of these liposomes were increased with time (days), while incorporation of cholesterol 25mol % to the liposomes (PC: cholesterol) reduce the temperature's effects and the optical densities show a very slight change with time (days) because cholesterol in high concentrations prevent the phospholipids packing and induce orientation and more rigidity to those phospholipids and therefore prevent liposome aggregation, liposomes prepared by using combinations of some lipids follows the order of physical stability form the correlation of the mean volume diameter, zeta potential and pH, egg lecithin (PC)/cholesterol (CH)/stearylamine (SA) < PC/CH/phosphatidylserine (PS) < bovine brain ceramides (CM)/CH/palmitic acid (PA)/CS < PC/CH/cholesteryl sulphate (CS) at 4°C, as well as at 25°C, after a 6-month storage period<sup>21</sup>. Panayiotou<sup>22</sup> studied the Liposome stability during incubation in presence of cyclodextrins (CDs) is studied. Dried-rehydrated vesicle (DRV), multilamellar vesicle (MLV) and small unilamellar vesicle (SUV) calcein-encapsulating liposomes, composed of different lipids are formulated, and retention of calcein is followed during vesicle incubation in hydroxypropyl- $\beta$ -CD (HP $\beta$ -CD), HP $\gamma$ -CD or methyl- $\beta$ -CD (Mep-CD), for 24 h. Results demonstrate that liposome integrity in cyclodextrins is affected by lipid composition and type. For the same lipid composition calcein release from vesicles is faster in the order: MLV > DRV > SUV. Mep-CD influences liposome stability most, compared to the other CD's studied. Vesicles composed of saturated phospholipids were found more stable compared to phosphatidyl-choline (PC) liposomes, suggesting that phospholipid saturation and membrane rigidity influences the interaction between liposomal-lipids and CD molecules. Chol (cholesterol) addition in lipid membrane improves PC-liposome integrity, but has opposite or no effect on liposomes consisting of saturated lipids. Decrease of vesicle dispersion turbidity and size distribution in presence of CD, implies that Mep-CD induces vesicle disruption and solubilization (to micelles). Turbidity measurements confirm that DRV liposomes are affected more than SUV. Charge on the liposomes also determines the in vivo stability; Liposomes with neutral charge containing phosphatidylcholine were the most stable and bound the lowest amount of protein. Liposomes with positive charge behaved similarly to those with neutral charge. However, the stability of negatively charged liposomes was very dependent on their composition. Those liposomes containing only one class of negatively charged phospholipids bound a great amount of protein and were very unstable. However, those liposomes containing phosphatidylcholine bound less protein and were more stable<sup>23</sup>.

### **Method of drug loading:**

Drug loading methods involves passive and active loading. Passive loading includes mechanical dispersion, solvent dispersion and detergent solubilization working on different principles. Remote or active loading method load drug molecules into preformed vesicles by using pH gradient and potential difference across liposomal membranes. The stability of the drug encapsulation was measured by various assays and it was found that according to the theoretical predictions<sup>24,25</sup>, active loading give the greater encapsulation efficiency. One which invention provides the encapsulation of weak acid compounds include ibuprofen, tolmetin, indomethacin, phenylbutazone, meclofenamic acid, piroxicam, ciprofloxacin, and nalidixic acid. Invention provides a method for loading a weak-acid compound into liposomes having a higher inside/lower outside pH gradient. Loading is carried out by adding the weak acid compound to a suspension of liposomes having a higher inside/lower outside gradient of a salt of a weak acid which includes the given cation. The protonated form of the weak acid salt acts as an inside-to-outside proton shuttle to generate a higher inside/lower outside pH gradient to drive loading of the weak acid compound into the liposome interior<sup>26</sup>. Dos Santos prepared the cholesterol free liposomes by standard pH gradient loading based method, that rely on incubation temperatures above the phase transition temperature ( $T_c$ ) of the bulk phospholipid to promote drug loading. In the absence of cholesterol, liposome permeability is enhanced at these temperatures which, in turn, can result in the collapse of the pH gradient and/or unstable loading. Doxorubicin loading studies, for example, indicate that the drug could not be loaded efficiently into cholesterol-free DSPC liposomes. this problem could be circumvented by the addition of ethanol as a permeability enhancer. Doxorubicin loading rates in cholesterol-free DSPC liposomes were 6.6-fold higher in the presence of ethanol. In addition, greater than 90% of the added doxorubicin was encapsulated within 2 h at 37 °C, an efficiency that was 2.3-fold greater than that observed in the absence of ethanol. Optimal ethanol concentrations ranged from 10% to 15% (v/v) and these concentrations did not significantly affect liposome size, retention. Most important, the stability of the imposed pH gradient. Ethanol-induced increased drug loading was temperature-, lipid composition- and lipid concentration-dependent. Results suggest that ethanol addition to preformed liposomes is an effective method to achieve efficient pH gradient-dependent loading of cholesterol-free liposomes at temperatures below the  $T_c$  of the bulk phospholipid<sup>27</sup>. Ammonium sulfate gradients loading gives rise to more stable drug retention was observed with encapsulation of brucine alkaloid into the soybean phosphatidylcholine (SPC) and hydrogenated soybean phosphatidylcholine (HSPC)<sup>28</sup>.

**Prodrug:**

Physical properties of drug cargo play a role in the stability and hence drug delivery kinetics of liposomal drug delivery system. Prodrug is another approach to enhance the drug retention stability. Problems like poor entrapment efficiency and physical as well as chemical instability have been found to be associated with the liposomal entrapment of drug molecules, other than those that are highly lipophilic<sup>29</sup>. Prodrug lipophilic character improves the interaction with lipid bilayers, favoring the absorption through the lipid barriers by allowing liposomes to work as a lipophilic carrier which is able to deliver drug near to the cell surface. Only care we have to take is that hydrophobic acyl chains anchored to the drug must match to acyl chains of lipids. Water-soluble antineoplastic agent 5-fluoro-2'-deoxyuridine (FUdR) encapsulated in water phase. Retention was assessed with plasma, storage upon refrigeration, diffusion of FUdR across liposomes faster in fluid type liposomes than lipophilic prodrug FUdR-dipalmitate and FUdR-dioctanoate<sup>30</sup>. Interestingly it has been observed that, a water soluble dye fluorescein was more stable than lipophilic rhodamine in distearylphosphatidylcholine than dipalmitoylphosphatidylcholine at 25 and 37° C. We can conclude that fine-tuning of lipid composition is also equally responsible for stability of liposomes along with partition behavior of drug.

**Pro-liposomes:**

In dispersed aqueous system liposomes have a problem of degradation by hydrolysis or oxidation as well as sedimentation, aggregation, or fusion of liposomes during storage. Other problem associated with clinical application of liposomes includes difficulties in large-scale production to obtain a product with adequate physical and chemical stability. In formulation of pro-liposomes lipid dried over a fine particulate, water soluble support like sodium chloride, sorbitol or polysaccharides imparts adequate physical and chemical stability and are ideally suitable for lipophilic drugs<sup>31</sup>.

**Lyophilization:**

The problems related to lipid oxidation and hydrolysis during shelf life of the liposomal product can be reduced by the storage of liposomal dispersion in the dry state. Changes in the arrangement of phospholipids within the bilayer of small vesicles have been attributed to the loosely packed head groups and tightly packed alkyl chains in the outer layer with the opposite arrangement in the inner layer of the bilayer<sup>32</sup>. This leads to a thermodynamically unstable state, which favors aggregation and/or fusion of the vesicles to counteract this instability<sup>32</sup>. Therefore, it is clear that the

composition of the lipid bilayer and the aqueous phase, the amount of external water, bilayer–drug interaction, and the storage conditions of the liposomal formulations will all influence liposome stability<sup>33,34,35</sup>. To circumvent these problems and achieve long-term stability of liposomes, freeze-drying (Lyophilization) has been used as an effective approach to render liposomes stable without compromising their physical state or encapsulation capacity<sup>24</sup>. However, freeze-drying of liposome systems without appropriate stabilizers will again lead to fusion of vesicles; a factor exploited by the dehydration–rehydration method<sup>34</sup>. To promote vesicle stability during the freeze-drying process, cryoprotectants, including saccharides (e.g. sucrose, trehalose, and lactose) and their derivatives, are employed<sup>35</sup>. A detailed review by Crowe and Crowe<sup>36</sup> outlines the mechanism of stabilization offered by the saccharides. Briefly, the increase in vesicle size and leakage of the encapsulated material in non-cryoprotected liposomes during freeze-drying is attributed to two factors: phase separation of lipids around their gel–fluid transition temperature and fusion of the membranes of the liposomes. Thus, aggregation of liposomes could be prevented by the formation of stable boundaries between the vesicles. The ability of cryoprotectants to form these stable boundaries has been attributed to their ability to replace the bound water around the bilayer via interaction with the polar region of the lipid head group (water replacement hypothesis<sup>36</sup>). Alternatively, Koster et al.<sup>37</sup> proposed the formation of a vitreous layer (glass formation around the bilayer) of the cryoprotectant around the bilayer, which depresses the transition temperature of the phospholipids thus preventing any drug leakage during gel to fluid phase transformations. The role of various saccharides (glucose, sucrose, trehalose, etc.) has been investigated thoroughly as stabilizing cryoprotectants<sup>38,39,40,41,42,43</sup>. Despite these detailed investigations, these systems have not been fully optimized, and in order to achieve effective cryoprotection and reproducible formulation parameters, high concentrations (up to 30 mol.% in some cases) are required that not only compromise the viscosity of the formulation post-hydration, but may also trigger unacceptable adverse reactions (e.g. in diabetic patients<sup>36</sup>). Crowe et al.<sup>37</sup> investigated amino acid proline as cryoprotectants, and Sterberg and Wadsten<sup>44</sup> who studied histidine, amino acids provide the added advantage of not only being able to offer hydrogen bonds, but also can provide electrostatic interactions for effective lyophilisation. The current work focuses on basic, polar amino acids, which include arginine, lysine and histidine<sup>45</sup>. Study by Hinrichs W.L.J et al.<sup>46</sup> study illustrate that various PEGylated liposomes can be stabilized by oligosaccharides provided that the oligosaccharide is compatible with PEG, inulins, which is

compatible with PEG, were capable to prevent aggregation. By contrast, dextran is incompatible with PEG and its incompatibility increase with increase in molecular weight. Recently, many studies showed that the addition of tertiary butyl alcohol (TBA) can considerably enhance the rate of ice sublimation, resulting in short drying cycles of sucrose solutions<sup>47,48,49</sup>. Therefore; it is desirable to freeze-drying of liposomes using TBA/water cosolvent systems if for economy concerns. Although freeze-drying of liposome-forming lipids from TBA (or aqueous TBA) has been successfully applied to prepare liposomes<sup>50,51</sup>. JingXia Cui et.al<sup>52</sup> concludes that it is possible to produce dehydrated hydrogenated soybean phosphatidylcholine (HSPC) liposomes by means of freeze-drying of HSPC liposomes with TBA/water cosolvent system. The addition of a small amount of TBA does not have any obvious influences on the HSPC vesicle size and the retention of trapped calcein, but also can result in short freeze-drying cycles. Moreover, freeze-drying of HSPC liposomes from TBA/water cosolvent systems can provide sterile powder for specialized applications. In conjunction with a modified alcohol injection method, this technology may be used to produce dehydrated HSPC liposomes on a large scale. The liposomal dispersions could be stored at room temperature in the freeze-dried form with minimum effect on stability, drug content and physicochemical properties. The freeze-dried lyophilized powder could be used for incorporation of liposomes in topical gel formulation (in powder form or reconstituted dispersion form) or could be reconstituted immediately prior to injection or further formulation processing.

#### **Electrosteric stabilization:**

The first description of sterically stabilized liposomes was reported in 1987-1988. Stable liposome suspensions require a repulsive interaction that is at least comparable to the magnitude and range of the Vander Waals force. The electrostatic repulsion can be realized by increasing the charge of a surface by lowering the ionic strength or altering the pH away from the point of charge of the solid or addition of charged molecules in the bilayer, which shift the electrophoretic mobility. A (very big) disadvantage of electrostatic stabilization is that it is highly sensitive with respect to surface charge (pH) and salt concentration. Another way, steric stabilization can be achieved by covering the surface with an adsorbed layer of long, bulky molecules to prevent the close approach of the liposomes. The combination of electrostatic and steric stabilization, which has been termed electrosteric stabilization (Fig.3), leads to highly stable dispersions<sup>53</sup>. The electrostatic component results from a net charge on the liposome surface and/or the charge associated with the adsorbed polymer. The steric component arises from the chain-chain exclusion of adsorbed polymeric material on

one surface from the other surface. Addition of other interactions expands the DLVO model to an extended DLVO inter-particle potential. For the most general case, the total potential between liposomes should include all the forces, including van de Waals attraction ( $V_{vdw}$ ), electrostatic repulsion ( $V_{elec}$ ), hydrophobic attraction ( $V_{hfo}$ ), hydration ( $V_{hyd}$ ), undulation ( $V_{und}$ ), steric repulsion ( $V_{st}$ ), repulsive protrusion forces ( $V_{pr}$ ), and attractive ion correlation forces ( $V_{icf}$ )

$$V_{tot} = -V_{vdw} - V_{hfo} - V_{icf} + V_{elec} + V_{hyd} + V_{und} + V_{st} + V_{pr}$$

Liposomes bound or coated with ligands, such as PEG, Carbopol 1342, polyvinyl alcohol, o-palmitoylpullulan, poloxamer 338, poly (vinylpyrrolidone), dicetyl phosphate (negative charge), stearylamine (positive charge) carboxymethylchitin or block copolymers have been investigated and shown to increase the liposome stability and control the drug release<sup>24</sup>. In case of polyvinyl alcohol, comparing with PEG-coated liposomes, the liposomes with PVA addition to the bilayer were more stable, and had higher entrapment efficiency. However, PEG is the most widely used stabilizer in pharmaceutical applications because of its advantages including non-biodegradability, high solubility in aqueous solution, ability to bind a large number of water molecules, high flexibility of its polymer chain, immunogenicity, antigenicity and limited accumulation in cells of the reticular endothelial system (RES)<sup>54</sup>. In addition, it has been shown that PEG could prevent the adsorption of proteins (opsonins) responsible for phagocytic removal, and provides the ability to independently adjust the liposome physicochemical properties (such as drug loading and leakage) and biological properties (e.g. blood circulation and tissue distribution). The term 'steric protection' is used with regard to liposome interaction with 'opsonins'. We should note that the term 'steric protection' is not self-explanatory. The 'steric' is as vague as "having to do with spatial arrangement if the atoms in the molecule" Ceh Boris [55] discussed the development of an effective anti-cancer liposomal formulation-doxorubicin in sterically stabilized liposomes. Doxorubicin in sterically stabilized, liposomes (Doxil™ by Sequus Pharmaceuticals, Inc., Menlo Park, CA) was approved by Food and Drug Administration and is commercially available since late 1995.

Papisov M I<sup>56</sup> in his paper discussed that, the relationships between liposome structure and circulation with respect to the theoretical mechanistic models of mass transfer, liposome interactions with cells and blood proteins, and boundary effects resulting from surface modification. Special attention was paid to the practical application and limitations of the models. Sadzuka Y et al.<sup>57</sup> results of experiment conclude that the mixed PEG –modification on the surface of liposomes

gave the increase of fixed aqueous layer thickness (FALT) increase of FALT and improvement of blood circulation in the blood, involvement of antitumor activity of DOX of these liposomes was suggested. The PEGylated, freeze-dried liposomal paclitaxel showed improved solubility and stability in comparison to current Taxol formulation. PEGylated liposomes increased the biological half-life of the paclitaxel from 5.05(±1.52) h to 17.8(± 2.35) h compared to conventional liposomes in rats. Biodistribution studies in breast cancer xenografted nude mouse model showed that PEGylated liposomes significantly decreased the uptake in Reticulo Endothelial System (RES)-containing organs (liver, spleen and lung) while increasing the uptake in tumor tissues after injection compared to Taxol or the conventional liposomal formulation. The PEGylated liposome showed greater tumor growth inhibition effect in *in vivo* studies. Therefore Yang Tao et.al.<sup>58</sup> concludes that, PEGylated liposomal formulation of paclitaxel could serve as a better alternative for the passive targeting of human breast tumors. Recently Ghosh PC and Rathore<sup>59</sup> studied the effect of surface charges and density of PEGylated lipid on the cytotoxicity of liposome entrapped ricin. Alternative sterically stabilizing polymer based on poly (amino acid) s(PAA)s in particular ,poly(hydroxyethyl L-glutamine)(PHEG) and poly(hydroxyethyl L-asparagine) (PHEA),both coupled to hydrophobic anchor to graft them onto the liposome bilayer showed prolonged circulation time ,comparable to those reported for PEGylated liposomes.A major advantage of PAAs is that they are enzymatically degraded, thereby reducing the risk of accumulation *in vivo* like other liposome

systems, they appear to activate the complement system<sup>60</sup>. Some novel approaches like, lectin modified liposomes have been studied but they are found susceptible to the fusion and coalescence than PEGylated liposomes. It was also observed that o-palmitoyl anchored carbohydrates stabilize the liposomes at different temperature and relative humidity conditions<sup>61</sup>. Coating with biocompatible polyelectrolyte like poly (lysine)(Pll), poly (glutamic acid)(Pga) polypeptides have been found to imparts positive and negative charge respectively<sup>62</sup>.

#### **Prevention from oxidation and hydrolysis:**

There are three steps in lipid oxidation: propagation of a single double bond, hydro peroxide decomposition, and bond breakdown and acetaldehyde formation. Numbers of factors are responsible for chemical instability of liposomes like pH, ionic strength and exposure to oxygen<sup>63,64</sup>. The better ways to minimize use of unsaturated lipids, use of argon or nitrogen environment to minimize the exposure to oxygen, use of antioxidants like  $\alpha$ -tocopherols, betahydroxy toluene (BHT) or nitroxides<sup>65</sup> (5 mol %) or use of light resistant container for the storage of liposomal formulations<sup>31</sup>. It was observed that cholesterol also protect liposomal lipids by reducing lipid bilayer hydration. Tempol a water soluble antioxidant better than the  $\alpha$ -tocopherol on long term studies<sup>66</sup>. This method is also applicable for stabilization of niosomes<sup>67</sup>.

#### **PATENTS RELATED WITH STABILITY OF LIPOSOMES:**

Refer table No. 2 for the same.

**Table 2: Patents related with stability of liposomes.**

Sr. No.	Titles of patents	Description of Invention
1	Liposome complexes for increased systemic delivery <sup>68</sup>	This invention relates with composition and method of preparation will form the stable Liposomes. Liposomes have been prepared for biologically active compounds referred as molecules that affect the biological system. These includes proteins, nucleic acids, therapeutic agents, vitamins and their derivatives, viral fractions, lip polysaccharides, bacterial fractions and hormones and other particular interest are chemotherapeutic agents. The liposomes of present invention carry net +/- charge when complexed with biologically active agents. The liposomes comprise a cationic lipid, DOTAP and cholesterol or a cholesterol derivative. More preferably, the molar ratio of DOTAP to cholesterol is 1:1-3:1. The liposomal composition of the present invention has shown to be very stable in a biological environment. An improved method for preparing the liposomes of the present invention employs sonication, heating, and extrusion. The extruded liposomes form sandwich liposomes whereas the non-extruded liposomes do not form such structures. Protein expression from nucleic acid: sandwich liposomes was approximately 2-fold higher as compared to MLVs.

2	Oral delivery of vaccines using polymerized liposomes <sup>69</sup>	This invention relates to the synthesis, preparation, and use of polymerized liposomes as sustained drug and/or antigen release devices. The polymerized liposome are generally prepared by polymerization of double bond-containing monomeric phospholipids, free radical polymerization (hydrophobic free radical initiator, such as AIBN (azo-bis-isobutyronitrile), or a hydrophilic free radical initiator such as AIPD (azo-bis-amidinopropane dihydrochloride) and radiation source, such as ultraviolet or gamma radiation. biologically active substance is entrapped prior to the polymerization of the monomeric double bond-containing liposome. Liposomes has been orally targeted by using bioadhesive ligands include attachment peptides such as RGD, and extracellular matrix materials such as fibronectin.
3	Method for improving stability and shelf-life of liposome complexes <sup>70</sup>	This invention relates with A method for preparing a stable cell-targeting complex comprising a ligand and a cationic liposome encapsulating a therapeutic or diagnostic agent comprises (a) combining the complex with a solution comprising a stabilizing amount of sucrose and (b) lyophilizing the resultant solution to obtain a lyophilized preparation; wherein, upon reconstitution, the preparation retains at least about 80% of its pre-lyophilization activity.
4	Method of making liposomes with improved stability during drying <sup>71</sup>	This invention relates generally to making liposomes that are stable during drying by freeze drying technique which may contain drugs or other therapeutic agents, diagnostic agents, or other materials of biological or pharmacological interest. In the preferred embodiment, the liposomes are made in the presence of the combination of at least one sugar (sucrose, trehalose, lactose, maltose and glucose) and at least one protein (gelatin and casein), polypeptide and/or oligopeptide, whereby upon reconstitution of the dried stabilized liposome, the liposome bilayer is maintained, aggregation or fusion is avoided, and the degree of drug encapsulation achievable on reconstitution is greatly improved.
5	Stabilization of photosensitive materials <sup>72</sup>	In this invention protective effect may be achieved by using multilamellar vesicles comprising a hydrophilic cyclodextrin capable of forming an inclusion complex with the photosensitive material in the aqueous phase and a combination of light absorbing (chemical absorbers and physical blockers of UV radiation) and antioxidant agents in the lipid bilayer (beta carotene quench both singlet oxygen driven photochemical reactions and free radical reactions).
6	Method of amphiphatic drug loading in liposomes by pH gradient <sup>73</sup>	It deals with an improved simple, efficient, safe, economical, and fast transmembrane loading procedure for efficient active loading of weak amphiphatic drugs into liposomes using the transmenbrane gradient. The resulting liposomes loaded with the amphiphatic drug are stable and safe. A storage able form of loadable liposomes has extended period of stability. The reversed procedure is applicable for sustained release of liposome encapsulated drugs from ammonium liposomes.
7	Liposomal compositions for parenteral delivery of agents <sup>74</sup>	The invention provides, combining the agent with a micelle-forming compound to form a micelle comprising the agent, wherein the agent is releasable from said micelle-forming compound and adding the micelle to the liposome, wherein the micelle combines with the liposome such that the agent is loaded into the liposome to form a loaded liposome.

## CONCLUSIONS

Wide research is necessary on stability of liposomes. During formulation to achieve good physical, chemical and biological stability we have to concentrate on number of methods according to type of drug delivery, properties of incorporated material etc. There is need to develop specific guidelines for stability testing of liposomes this would facilitate the application of ribozymes, antisense oligonucleotides, Triple helix forming oligonucleotides, short interfering RNAs and other drugs to the therapy of infectious diseases and cancer.

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