A Review on Liposomes as a Topical Drug Delivery
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ABSTRACT

In this article information about various methods of liposome preparation (including the large scale manufacture) has been reviewed. The advantages and disadvantages of the methods have been described in terms of size distribution and encapsulation efficiency. Liposomes are simple microscopic vesicles in which lipid bilayer structures are present with an aqueous volume entirely enclosed by a membrane, composed of lipid molecule. There are a number of components present in liposomes, with phospholipid and cholesterol being the main ingredients. Liposomes having concentric phospholipid bilayers molecules from low molecular weight (glucose) to high molecular weight (peptides and proteins) have been incorporated in liposomes. The water soluble compounds/drugs are present in aqueous compartments while lipid soluble compounds/drugs and amphiphilic compounds/drugs insert themselves in phospholipid bilayers. The type of phospholipids includes phosphoglycerides and sphingolipids, and together with their hydrolysis products. Classification of liposomes is based on lamellae and composition and on the basis of size and number of lamellae.

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INTRODUCTION

Liposomes are microscopic vesicles composed of one or more lipid bilayers arranged in concentric fashion enclosing an equal number of aqueous compartments. Various amphipathic molecules have been used to form the liposomes and the method of preparation can be tailored to control their size and morphology. Drug molecules can either be encapsulated in the aqueous space or intercalated into the lipid bilayer; the exact location of a drug in the liposome will depend upon its physicochemical characteristics and the composition of the lipids.[1-4]

Liposomes, i.e., phospholipid vesicles, are widely applied for the topical treatments of diseases in dermatology. Many drugs encapsulated into liposomes show enhanced skin penetration. Because of their ability to provide a sustained and controlled release of the incorporated material, liposomes also have a potential for being applied vaginally. The major disadvantage of using liposomes topically and vaginally lies in the liquid nature of the preparation. To achieve the viscosity desirable for application, liposomes should be incorporated into a suitable vehicle. It has been well established that liposomes are fairly compatible with viscosity increasing agents (methylcellulose) and polyacrylic acid (Carbopol).[5, 6]

Several techniques have been explored to increase the drug penetration rate across skin including iontophoresis and penetration enhancement, particularly for the delivery of peptides and proteins. Here focus on a third alternative method, the encapsulation of drugs in lipid vesicles prepared from phospholipids (liposomes) which have been shown to facilitate transport of drugs into and across skin. While liposomes have been investigated for many years as parenteral drug carrier systems, particularly for the selective delivery of anticancer, antibiotic and antifungal agents, they have only for approximately one decade been considered for topical drug delivery, including ophthalmic, pulmonary and dermal/transdermal delivery.[7-9]

Due to their high degree of biocompatibility, liposomes were initially conceived of as delivery systems for intravenous delivery. It has since become apparent that liposomes can also be useful for delivery of drugs by other routes of administration. Liposomes are frequently used as vehicles in pharmaceuticals and cosmetics for a controlled and optimized delivery to particular skin layers. Liposomes are spherical vesicles whose membrane consists of amphiphilic lipids (i.e., lipids that are hydrophilic on one side and lipophilic on the other side) that enclose an aqueous core, similar to the bilayer membranes of living cells. Because liposomes offer an amphiphilic environment, they may encapsulate hydrophilic substances in their aqueous core and lipophilic substances in their lipid bilayer. This unique dual release capability enables the delivery of 2 types of substances once they are applied on the skin; each differs in its effects on skin permeability, which may enhance the desired therapeutic benefit.[10]

Structure and Composition of Liposome:

Among the variety of new drug delivery systems. Liposomes seem to have the best potential to accommodate both water and lipid soluble compounds, to protect the liposome-encapsulated drug from metabolic degradation and to act as a delivery mechanism, releasing active ingredients slowly and in a controlled manner. Phospholipids, the cornerstone of the liposome lipid bilayer. Usually extracted from egg yolk or soy bean oil consist of a hydrophilic head portion covalently attached to two hydrocarbon tails representing the lipophilic portion. Aggregation in a bilayer structure occurs by orientation of the hydrophilic head groups towards the aqueous environment. While keeping the lipophilic hydrocarbon chains sequestered inside. Formation of such a configuration provides the vesicle with the lowest potential energy state through solvation of the polar head groups and hydrophobic interactions of the lipid chain.[10, 11] Natural phosphatidylcholine extracted from egg yolk or soy bean oil or its semisynthetic derivatives represents the main constituent in various liposomal formulations. The chemical structure of naturally occurring
phosphatidylcholine has a glycerol moiety attached to two acyl chains which may be saturated or unsaturated. Each may have between 10 to 24 carbon atoms. Together forming the hydrophobic (lipophilic) portion of the molecule. The charged phosphate and choline moieties form the hydrophilic “head”. The fatty acid chains, depending on their length and degree of saturation, can exist in the gel phase in which the lipids are rigid, impermeable and easily aggregated upon storage or in the more fluid liquid-crystalline phase. The temperature at which the gel phase converts to the liquid crystalline phase is known as the transition temperature. Cholesterol is frequently added in minute quantities to most liposomal formulations to increase the fluidity of the liposomal gel phase enhance the retention of hydrophilic particles and to stabilize the bilayer membrane in a manner similar to that of biological membranes.\[10, 12\]

Advantage of topical liposome why is there any need for new drug carrier systems with topical dermatics?

The major problem concerning the efficacy of topical drugs is that they have to reach the site of action and to stay there in an effective concentration for a certain time. Although the skin belongs to the organs which can be reached directly drug application on the skin surface does not automatically mean the drug getting to the right site of action. This, in fact, is the problem with the conventional dosage forms like creams and ointments. The use of penetration enhancers, e.g. dimethyl sulphoxide (DMSO) or propylene glycol leads, on the one hand, to an improved transport rate through the epidermal barrier but, on the other, to more unwanted effects due to an increased systemic drug level. Moreover, irritative or even toxic side effects are reported leading to the conclusion that addition of penetration enhancers does not really mean an improvement in topical drug administration.\[1,14\]

What are the advantages of liposomes as drug carrier systems?

1. Similar to biological membranes they can store water-soluble and lipophilic substances in their different phases i.e. it readily incorporate a wide variety of hydrophilic and hydrophobic drugs.
2. They are similar to the epidermis with respect to their lipid composition which enables them to penetrate the epidermal barrier to a greater extent compared to other dosage forms.
3. According to studies performed so far liposomes are biodegradable and non-toxic which is important to avoid side effects.\[3\]
4. The really new aspect with liposomes is that they are thought to act as “drug localizers” - not only as “drug transporters”. i.e. to enhance significantly the accumulation of drug at the site of administration as a result of the high substantivity of liposomes with biological membranes.
5. The uptake of intact liposomes by the reconstructed epidermis, these vesicles do not penetrate through healthy skin. Yet, this is to be expected in diseased skin without intact epidermal barrier. This is especially important as far as drugs like glucocorticosteroids or retinoids are concerned which are known to create severe systemic effects when absorbed percutaneously to a greater extent. Vehicles which can transport these drugs to the wanted site of action within the skin would thus prevent systemic absorption and consecutively unwanted effects. This is the reason why liposomes as a promising form for topical drug delivery.
Classification of Liposomes:

Table 1: Classification of liposomes based on size and lamellarity\textsuperscript{[13]}

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Size Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV</td>
<td>Multilamellar large vesicles</td>
<td>(&gt;0.5 µm)</td>
</tr>
<tr>
<td>OLV</td>
<td>Oligolamellar vesicles</td>
<td>(0.1–1 µm)</td>
</tr>
<tr>
<td>UV</td>
<td>Unilamellar vesicles</td>
<td>(all sizes)</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
<td>(20–100 nm)</td>
</tr>
<tr>
<td>MUV</td>
<td>Medium-sized unilamellar vesicles</td>
<td>-</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
<td>(&gt;100 nm)</td>
</tr>
<tr>
<td>GUV</td>
<td>Giant unilamellar vesicles</td>
<td>(&gt;1 µm)</td>
</tr>
<tr>
<td>MVV</td>
<td>Multivesicular vesicles</td>
<td>(usually &gt;1 µm)</td>
</tr>
</tbody>
</table>

Table II: Based on Method of Liposome preparation\textsuperscript{[10,13]}

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>REV</td>
<td>Single or Oligolamellar vesicles made by Reverse phase Evaporation method</td>
</tr>
<tr>
<td>MLV-REV</td>
<td>Multilamellar vesicles made by Reverse phase Evaporation method</td>
</tr>
<tr>
<td>SPLV</td>
<td>Stable plurilamellar vesicles</td>
</tr>
<tr>
<td>FATMLV</td>
<td>Frozen and Thawed MLV</td>
</tr>
<tr>
<td>VET</td>
<td>Vesicles prepared by extrusion technique</td>
</tr>
<tr>
<td>DRV</td>
<td>Dehydration-Rehydration Method</td>
</tr>
</tbody>
</table>

Method of preparation and drug loading:

Liposomes are manufactured in majority using various procedures in which the water soluble (hydrophilic) materials are entrapped by using aqueous solution of these materials as hydrating fluid or by the addition of drug/drug solution at some stage during manufacturing of the liposomes. The lipid soluble (lipophilic) materials are solubilized in the organic solution of the constitutive lipid and then evaporated to a dry drug containing lipid film followed by its hydration. These methods involve the loading of the entrapped agents before or during the manufacturing procedure (Passive loading). However, certain type of compounds with ionizable groups, and those which display both lipid and water solubility, can be introduced into the liposomes after the formation of intact vesicles (remote loading).\textsuperscript{[14]}

Mechanical dispersion methods:

Preparation of liposomes by lipid film hydration:

Preparation of Lipid for Hydration:

The lipids must first be dissolved and mixed in an organic solvent to assure a homogeneous mixture of lipids. Usually this process is carried out using chloroform or chloroform: methanol mixtures. Typically lipid solutions are prepared at 10-20mg lipid/ml of organic solvent, although higher concentrations may be used if the lipid solubility and mixing are acceptable. Once the lipids are thoroughly mixed in the organic solvent, the solvent is removed to yield a lipid film. For small volumes of organic solvent (<1mL), the solvent may be evaporated using a dry nitrogen or argon stream in a fume hood. For larger volumes, the organic solvent should be removed by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask. The lipid film is thoroughly dried to remove residual organic solvent by placing the vial or flask on a vacuum pump overnight. The lipid solution is transferred to containers and frozen by placing the containers on a block of dry ice or
swirling the container in a dry ice-acetone or bath. After freezing completely, the frozen lipid cake is placed on a vacuum pump and lyophilized until dry. The thickness of the lipid cake should not be more than the diameter of the container being used for lyophilization. Dry lipid films or cakes can be removed from the vacuum pump, the container should be closed tightly and taped, and stored frozen until ready to hydrate.¹⁴

**Hydration of Lipid Film/Cake:**

Addition of the hydrating medium, the lipid suspension should be maintained above the Tc during the hydration period. For high transition lipids, this is easily accomplished by transferring the lipid suspension to a round bottom flask and placing the flask on a rotary evaporation system without a vacuum. Spinning the round bottom flask in the warm water bath maintained at a temperature above the Tc of the lipid suspension allows the lipid to hydrate in its fluid phase with adequate agitation. A hydration time of 1 hour with vigorous shaking, mixing, or stirring is highly recommended. Suitable hydration media accepted solutions which meet these conditions are 0.9% saline, 5% dextrose and 10% sucrose. During hydration some lipids form complexes unique to their structure. Highly charged lipids have been observed to form a viscous gel when hydrated with low ionic strength solutions. As particles approach one another there is no hydration repulsion to repel the approaching particle and the two membranes fall into an energy well where they adhere and form aggregates. The aggregates settle out of solution as large flocculates which will disperse on agitation but reform upon sitting. The product of hydration is a large, multilamellar vesicle (LMV) analogous in structure to an onion, with each lipid bilayer separated by a water layer. Once a stable, hydrated LMV suspension has been produced, the particles can be downsized by a variety of techniques, including sonication or extrusion.¹⁵

**Sizing of lipid suspension:**

**Sonication:**

Sonication of an LMV dispersion is accomplished by placing a test tube containing the suspension in a bath sonicator (or placing the tip of the sonicator in the test tube) and sonicating for 5-10 minutes above the Tc of the lipid. The lipid suspension should begin to clarify to yield a slightly hazy transparent solution. The haze is due to light scattering induced by residual large particles remaining in the suspension. These particles can be removed by centrifugation to yield a clear suspension of SUV. Mean size and distribution is influenced by composition and concentration, temperature, sonication time and power, volume, and sonicator tuning. Since it is nearly impossible to reproduce the conditions of sonication, size variation between batches produced at different times is not uncommon. Also, due to the high degree of curvature of these membranes, SUV are inherently unstable and will spontaneously fuse to form larger vesicles when stored below their phase transition temperature.¹⁵

**French pressure cell method:**

The method involves the extrusion of MLV at 20,000 psi at 4°C through a small orifice. The method has several advantages over sonication method. The method is simple, rapid, reproducible and involves gentle handling of unstable materials (Hamilton and Guo, 1984). The resulting liposomes are somewhat larger than sonicated SUVs. The drawbacks of the method are that the temperature is difficult to achieve and the working volumes are relatively small (about 50 mL maximum).¹⁶
Membrane extrusion liposomes:

Fig.-1: Liposomes Prepared By Membrane Extrusion Method

Dried reconstituted vesicles (drvs) and freeze thaw sonication (fts):

SUVs are rapidly frozen and followed by slow thawing. The brief sonication disperses aggregated materials to LUV. The formation of unilamellar vesicles is due to the fusion of SUV during the processes of freezing and or thawing. This type of fusion is strongly inhibited by increasing the ionic strength of the medium and by increasing the phospholipid concentration. The encapsulation efficiencies from 20 to 30% were obtained. [16]

Solvent dispersion methods:
Ether injection method:

A solution of lipids dissolved in diethyl ether 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 main drawbacks of the method are population is heterogeneous (70-190 nm) and the exposure of compounds to be encapsulated to organic solvents or high temperature. [17]

Ethanol injection method:

A lipid solution of ethanol is rapidly injected to a vast excess of buffer. The MLVs are immediately formed. The drawbacks of the method are that the population is heterogeneous (30-110 nm), liposomes are very dilute, it is difficult to remove all 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.

Reverse phase evaporation method:

First water in oil emulsion is formed by brief sonication of a two phase system containing phospholipids in organic solvent (diethylether or isopropylether or mixture of isopropyl ether and chloroform) and aqueous buffer. The organic solvents are removed under reduced pressure, resulting in the formation of a viscous gel. The liposomes are formed when residual solvent is removed by continued rotary evaporation under reduced pressure. With this method high encapsulation efficiency up to 65% can be obtained in a medium of low ionic strength for example 0.01M NaCl. The method has been used to encapsulate small and large macromolecules. The main disadvantage of the method is the exposure of the materials to be encapsulated to organic solvents and to brief periods of sonication. [18]
Detergent removal method:

The detergents at their critical micelles concentrations have been used to solubilize lipids. As the detergent is removed the micelles become progressively richer in phospholipid and finally combine to form LUVs. The detergents can be removed by dialysis. The advantages of detergent dialysis method are excellent reproducibility and production of liposome populations which are homogenous in size. The main drawback of the method is the retention of traces of detergent(s) within the liposomes. A commercial device called LIPOPREP (Diachema AG, Switzerland) which is a version of dialysis system is available for the removal of detergents. Other techniques have been used for the removal of detergents:
(a) by using Gel Chromatography involving a column of Sephadex G-25[19]
(b) by adsorption or binding of Triton X-100 (a detergent) to Bio-Beads SM-2[20]
(c) by binding of octyl glucoside (a detergent) to Amberlite XAD-2 beads.[21]

Industrial production of liposomes:

Of the several preparation methods described in the literature, only a few have potential for large scale manufacturing of liposomes. The main issues faced by formulator and production supervisor are presence of organic solvent residues, physical and chemical stability, pyrogen control, sterility, size and size distribution and batch to batch reproducibility. Liposomes for parenteral use should be sterile and pyrogen free. For animal experiments, adequate sterility can be achieved by the passage of liposomes through 400 nm pore size Millipore filters. For human use, precautions for sterility must be taken during the entire preparation process: that is,
1. The raw materials must be sterile and pyrogen free,
2. Preparation in sterile system: working areas equipped with laminar flow.
3. Use of sterile containers.

Some issues related to phospholipids need attention.[22, 23] The liposomes based on crude egg yolk phospholipids are not very stable. The cost of purified lipids is very high. [24] The liposomes prepared from polymerizable phospholipids are exposed to UV light. The polymerization process takes place in the bilayer(s). Such lipid preparations usually have better storage stability. It should be noted that such materials usually are phospholipid analogues and their metabolic fates have yet to be established.

Detergent Dialysis:

A pilot plant under the trade name of LIPOPREPR II-CIS is available from Diachema, AG, Switzerland. The production capacity at higher lipid concentration (80 mg/ml) is 30 ml liposomes/minute. But when lipid concentration is 10-20 mg/ml then up to many liters of liposomes can be produced. In USA, LIPOPREPR is marketed by Dianorm-Geraete.[25]

Microfluidization:

A method based on microfluidization/microemulsification/homogenization was developed for the preparation of liposomes. MICROFLUIDIZER is available from Microfluidics Corporation, Massachusetts, USA. A pilot plant based on this technology can product about 20 gallon/minute of liposomes in 50-200 nm size range. The encapsulation efficiency of up to 75% could be obtained.[25] Aqueous dispersions of liposomes often have tendency to aggregate or fuse and may he susceptible to hydrolysis and or oxidation. Two solutions have been proposed:
Proliposomes:
In proliposomes, lipid and drug are coated onto a soluble carrier to form free-flowing granular material which on hydration forms an isotonic liposomal suspension. The proliposome approach may provide an opportunity for cost-effective large scale manufacturing of liposomes containing particularly lipophilic drugs.[26]

Lyophilization:
Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at extremely low pressures. The technique has a great potential as a method to solve long term stability problems with respect to liposomal stability. It is exposed that leakage of entrapped materials may take place during the process of freeze-drying and on reconstitution. Recently, it was shown that liposomes when freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism) retained as much as 100% of their original contents. It shows that trehalose is an excellent cryoprotectant (freeze-protectant) for liposomes. Freeze-driers range in size from small laboratory models to large industrial units is available from Pharmaceutical Equipment Suppliers. These are characterized by a relatively short blood circulation time. To overcome this problem, long-circulating liposomes (also called stealth or sterically stabilized liposomes) have been developed. These stealth liposomes carry a polymer coating to obtain prolonged circulation times. Targeted liposomes (immunoliposomes) may be either conventionally or sterically stabilized and have specific antibodies or antibody fragments on their surface to enhance target site binding[26].

Mechanism of action:
A liposomal formulation to be effective, especially for hydrophilic drugs, it is essential that the suspension undergo significant dehydration. Since in most studies reported the lipid concentration scarcely exceeds 100 mg/ml, the bulk aqueous medium constitutes roughly 90% of the formulation. Thus, without a high degree of dehydration, no advantages over simple aqueous solution can be governed by employing liposomal systems, especially if the drug action is anticipated to occur within few hours after application. The dehydration of liposomal suspension can either be complete or reach an equilibrium stage wherein a certain amount of water is always held within the bilayers. Two interdependent factors control the extent of dehydration of a liposomal suspension.
1. The first is the phase transition temperature(Tm).
2. The second, often one that affects Tm, is the presence of components that either affect bilayer packing or those that are humectants /cytoprotectants, such a hydrophilic polymers or glycerol and sugars. The combined effect of the two factors will determine how much water will be retained by the liposomal bilayers following dehydration under non-occluded conditions. In the absence of enhancer effects arising out of the action of lipid components of the liposomal bilayers on skin, the extent and rate of dehydration of the liposomal bilayers control the extent and rate of transfer of drug, regardless of whether it is hydrophobic or hydrophilic, into skin.[27]

Transfer of hydrophobic drugs:
A major fraction of the added drug would be encapsulated or intercalated within the lipid bilayers of the liposomes. Further, optimum loading of hydrophobic drugs would be possible only if the lipid bilayers are maintained above the Tm of the major lipid. The transfer of drug from the lipid bilayers into skin can occur as long as the bilayers are in a liquid crystalline state. If the liquid crystalline phase is altered to the gel state, transport of the drug will cease or be negligibly low. Dehydration of liposomal suspensions has been shown to induce transitions from the liquid crystalline phase to the gel state. Thus, the extent of dehydration will
determine if changes in the state of the liposomal bilayers from a liquid crystalline phase to the gel state are possible. If dehydration is complete and the bilayers are transformed from the liquid crystalline state to the gel state, then transfer of drug from the bilayer to the skin ceases. If dehydration to an equilibrium stage wherein a constant amount of water is always retained in the bilayers occurs, then transport of drug would be continues and steady. A second consequence of dehydration involves the formation of a strong adhesive patch of liposomal bilayer on the skin. The formation of such patches maximizes the intimacy of contact between the drug-laden bilayer and the skin and probably is medicated via calcium bridges. [28]

**Transfer of hydrophilic drugs:**

The mode of action for liposomal transport of hydrophilic drugs parallels that for hydrophobic drug in qualitative manner. This is strictly because of major role of the water associated with the bilayers upon dehydration of the liposomal suspension. Thus, for liposomal systems that retain a constant amount of water within the bilayers following dehydration to an equilibrium slate, drug transport would continue over extended periods of time. A major consequence of dehydration for hydrophilic drugs involves the enhancement or enrichment of drug concentration in the aqueous phase of the bilayers leading to an enhancement influx of drug into and across skin. [29]

**The follicular option:**

When a follicular pathway is available, upon dehydration the liposomal bilayers can partition and pack into the follicular or hair ducts. This partitioning is favorable since the follicular ducts contain lipids. A prerequisite to the successful development of liposome products is the capability to scale-up production methods at acceptable costs using processes that provide a high degree of reproducibility required for the finished products. Here a mixture of lipids dissolved in a volatile organic solvent is deposited as a thin film on the surface of a round bottom flask as the solvent is removed under reduced pressure by rotary evaporation. The MLVs will form spontaneously when an excess volume of aqueous buffer is added to the dry lipid and the flask is hand shaken vigorously. Pro-liposome method, increase the surface area of dried lipid film by drying the lipid over finely devided particulate support, such as powdered sodium chloride. These dried lipid coated particulate are called pro-liposome. Injection of a water-immiscible solvent such as ether containing a mixture of bilayer-forming lipids into an aqueous medium at 55-65 °C or under reduced pressure will form single layer vesicles upon evaporation of the ether. The reverse phase evaporation technique is another method designed to form LUVs. A water-in-oil emulsion of phospholipids and buffer in an excess of organic phase is sonicated followed by removal of the organic phase under vacuum. Removal of the last traces of solvent transforms the emulsion from the gel state into large unilamellar vesicles. [27]

**Purification of Liposomes:**

Liposomes are commonly purified by either gel filtration column chromatography or by dialysis or centrifugation. In column chromatographic separation Sephadex G-50 is most widely used material. In this column chromatographic separation liposome membrane may bind or interact with the surface of the polydextran beads. There may be small amount of lipid lost resulting into destabilization of the membrane leading to permeability changes and subsequent leakage of entrapped solute. This problem can be overcome either by avoiding forming too small size liposomes of the same lipid composition as the test sample either before or after the packing of the column. [29]
Evaluation:

Size distribution

Prepared liposomal batches were monitored for their morphological attributes using optical microscope. Mean vesicle size and size distribution profile of liposome was determined by using Malvern particle size analyzer model SM 2000, which follows Mie's theory of light scattering. Diluted liposome suspension was added to the sample dispersion unit containing stirrer and stirred at 2000 rpm in order to reduce the interparticle aggregation, and laser obscuration range was maintained between 10-20%. The average particle size was measured after performing the experiment in triplicate. \[30\]

Entrapment efficiency:

Fluconazole associated with liposome was separated from unentrapped drug using centrifugation method. \[31\] Liposomes were centrifuged at 20000 rpm for 1 h at controlled temperature of 4 C. Supernatant containing unentrapped fluconazole was withdrawn and measured UV spectrophotometrically at 260 nm against phosphate buffer saline (pH 7.4). The amount of fluconazole entrapped in liposome was determined as follow:

\[
EE (\%) = \frac{([C_d - C_f]/C_d) \times 100}{100}
\]

Where, C is concentration detected of total fluconazole and C is concentration of free fluconazole. The entrapment efficiency was obtained by repeating the experiment in triplicate and the values were expressed as mean standard deviation.

Zeta potential (ζ) determination:

Charge on empty and drug loaded vesicles surface was determined using Zetasizer 300HSA (Malvern Instruments, Malvern, UK). Analysis time was kept for 60 s and average zeta potential and charge on the liposome was determined.\[30\]

Preparation of liposomal gel:

On the basis of factorial design approach, liposome batch (LPE) was selected for further formulation studies of liposomal gel. \[32\] Gel was prepared using carbopol® 934 NF (1, 1.5 and 2%). The appropriate quantity of carbopol 934 powder was dispersed into distilled water under constant stirring with a glass rod, taking care to avoid the formation of indispersible lumps and allowed to hydrate for 24 h at room temperature for swelling. Topical liposome gel formulations were prepared by incorporation of liposome's containing fluconazole (separated from the unentrapped drug) were mixed into the carbopol gel with a mechanical stirrer (25 rpm, 2 m). The dispersion was neutralized using triethanolamine (0.5% w/w). Control gels were made under the same conditions. \[33\]

Rheological studies:

While considering the stable liposome dispersion or any other delivery system they usually need to be incorporated into convenient dosage for to obtain formulation with desired semisolid consistency for ease in topical and transdermal application. It is important and controls the flow properties to ensure product quality and effectiveness of the production. \[34,35\] It helps in selection of dermatological formulation that will progress to clinical efficacy. In present study liposomal gels were prepared using carbopol 934 as gelling agent. Rheological analysis of liposome loaded carbopol gels were performed using a stress control rheometer (Viscotech Rheometer, Rheologica Instruments AB, Lund, Sweden), equipped with stress rheologic basic software, version 5, using cone-plate geometry with a diameter of the cone being 25 mm and a cone angle of
operating in the oscillation and static mode. Rheological analysis was performed at room temperature. The following parameters were carried out for rheology measurement.

**Oscillation stress sweep:**

Dynamic oscillation stress sweep was performed to determine the linear viscoelastic region (LVR). LVR is the region where the elastic modulus (G') was independent of applied stress because destruction in the structure of gels occurs at high shear stress. Analysis of viscoelastic material was designed not to destroy the structure so that measurement can provide the information about intermolecular and interparticle forces in the material. This test gives idea about the critical stress beyond which the sample may show significant structural changes, and therefore the consequent choice of the stress value to be used in other in other oscillation tests. The samples were exposed to increasing stress (0.5 to 150 Pa) at a constant frequency of 0.1 Hz. The three main parameters determined in this test were the storage modulus G', loss modulus G'' and loss tangent tan δ. The end point of the linear viscoelastic region was determined as a stress, when the G' value was dropped 10% from the linear level that indicated a significant change in the structure gel samples.

**Oscillation frequency sweep:**

The samples were exposed to stepwise increasing frequency (0.1 to 100 Hz) at a constant stress in the field of LVR and elastic moduli (G') as well as viscous modulus (G'') were recorded against frequency.

**Creep-recovery:**

The creep recovery test was used to determine the viscoelastic properties of the selected silk fibroin gel samples. The samples were exposed to the selected averaged stress of the stress sweep mode for 50 s. It was followed by relaxation period for 100 s for recovery. The creep compliance Jc (defined as the ratio of measured strain to the applied stress) was monitored against time.

**Drug content and content uniformity:**

The gel sample (100 mg) was withdrawn and drug (fluconazole) content was determined using UV spectrophotometer at 260 nm. Similarly, the content uniformity was determined by analyzing drug concentration in gel taken from 3 to 4 different points from the container. In case of liposomal gel, it was shaken with sufficient quantity of methanol to extract the drug and then analyzed by using UV spectrophotometer at 260 nm.

**Stability studies:**

The ability of vesicles to retain the drug (i.e., drug retentive behavior) was assessed by keeping the liposomal suspensions and liposomal gel at two different temperature conditions, i.e., 4-8 °C (Refrigerator; RF), 25±2 °C (Room temperature; RT), for a period of 60 days. Samples were withdrawn periodically and analyzed for the drug content and particle size for liposomal suspension and drug deposition for liposomal gel in the manner described under entrapment efficiency and particle size distribution studies.
REFERENCES


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