Preparation of Liposomes

Mechanism of Vesicle Formation

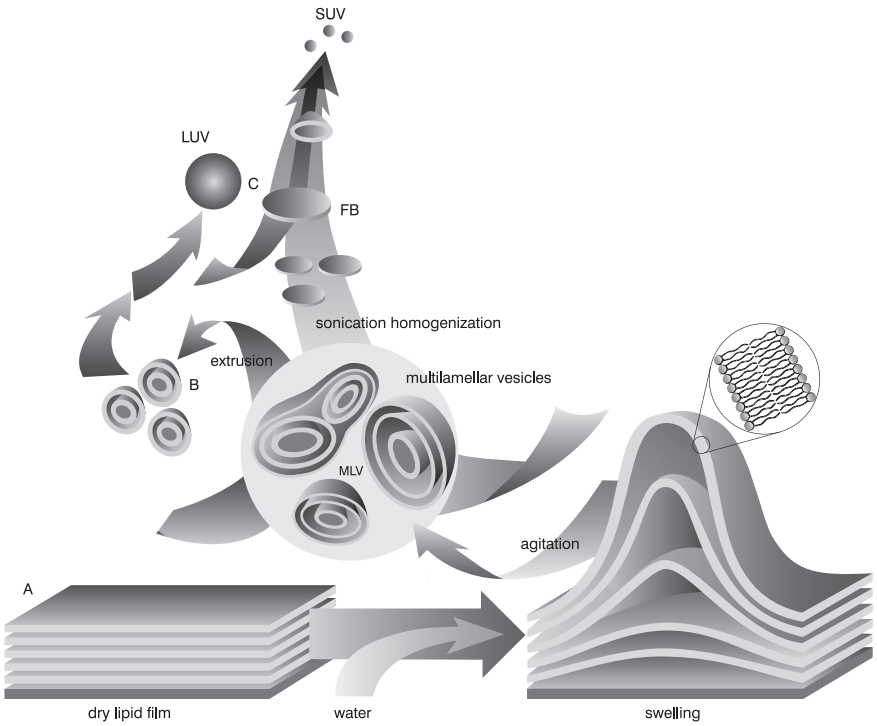
Liposomes (lipid vesicles) are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self-close to form large, multilamellar vesicles (LMV) which prevents interaction of water with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, reducing the size of the particle requires energy input in the form of sonic energy (sonication) or mechanical energy (extrusion).

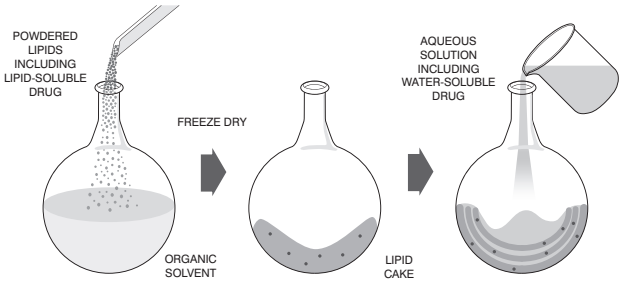
Method of Liposome Preparation

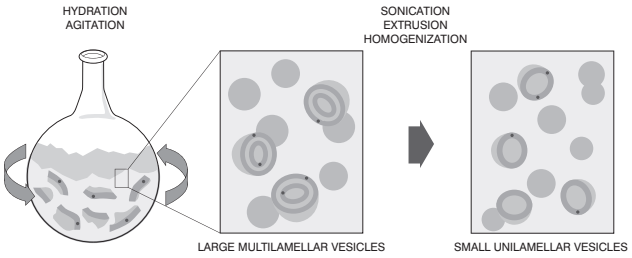
Properties of lipid formulations can vary depending on the composition (cationic, anionic, neutral lipid species), however, the same preparation method can be used for all lipid vesicles regardless of composition. The general elements of the procedure involve preparation of the lipid for hydration, hydration with agitation, and sizing to a homogeneous distribution of vesicles.

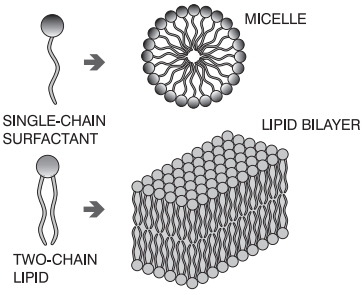
Preparation of lipid for hydration:

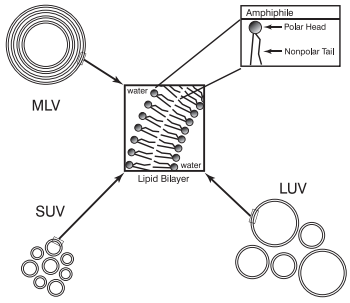
When preparing liposomes with mixed lipid composition, the lipids must first be dissolved and mixed in an organic solvent to assure a homogeneous mixture of lipids. Usually this process is carried out using chloroform or chloroform:methanol mixtures. The intent is to obtain a clear lipid solution for complete mixing of lipids. Typically lipid solutions are prepared at 10-20mg lipid/ml 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. If the use of chloroform is objectionable, an alternative is to dissolve the lipid(s) in tertiary butanol or cyclohexane. 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 alcohol (ethanol or methanol) bath. Care should be taken when using the bath procedure that the container can withstand sudden temperature changes without cracking. After freezing completely, the frozen lipid cake is placed on a vacuum pump and lyophilized until dry (1-3 days depending on volume). The thickness of the lipid cake should be no 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 closed tightly and taped, and stored frozen until ready to hydrate.

Hydration of lipid film/cake:

Hydration of the dry lipid film/cake is accomplished simply by adding an aqueous medium to the container of dry lipid and agitating. The temperature of the hydrating medium should be above the gel-liquid crystal transition temperature (T_c or T_m) of the lipid with the highest T_c before adding to the dry lipid. After addition of the hydrating medium, the lipid suspension should be maintained above the T_c 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 rotory evaporation system without a vacuum. Spinning the round bottom flask

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in the warm water bath maintained at a temperature above the T_c of the lipid suspension allows the lipid to hydrate in its fluid phase with adequate agitation. Hydration time may differ slightly among lipid species and structure, however, a hydration time of 1 hour with vigorous shaking, mixing, or stirring is highly recommended. It is also believed that allowing the vesicle suspension to stand overnight (aging) prior to downsizing makes the sizing process easier and improves the homogeneity of the size distribution. Aging is not recommended for high transition lipids as lipid hydrolysis increases with elevated temperatures.

The hydration medium is generally determined by the application of the lipid vesicles. Suitable hydration media include distilled water, buffer solutions, saline, and nonelectrolytes such as sugar solutions. Physiological osmolality (290 mOsm/kg) is recommended for in vivo applications. Generally accepted solutions which meet these conditions are 0.9% saline, 5% dextrose, and 10% sucrose.

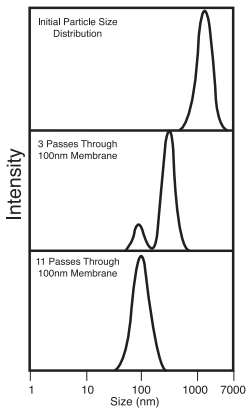
During hydration some lipids form complexes unique to their structure. Highly charged lipids have been observed to form a viscous gel when hydrated with low ionic strength solutions. The problem can be alleviated by addition of salt or by downsizing the lipid suspension. Poorly hydrating lipids such as phosphatidylethanolamine have a tendency to self aggregate upon hydration. Lipid vesicles containing more than 60 mol% phosphatidylethanolamine form particles having a small hydration layer surrounding the vesicle. 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 floculates 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. The spacing between lipid layers is dictated by composition with poly hydrating layers being closer together than highly charged layers which separate based on electrostatic repulsion. Once a stable, hydrated LMV suspension has been produced, the particles can be downsized by a variety of techniques, including sonication or extrusion.

Sizing of lipid suspension:

Sonication: Disruption of LMV suspensions using sonic energy (sonication) typically produces small, unilamellar vesicles (SUV) with diameters in the range of 15-50nm. The most common instrumentation for preparation of sonicated particles are bath and probe tip sonicators. Cup-horn sonicators, although less widely used, have successfully produced SUV. Probe tip sonicators deliver high energy input to the lipid suspension but suffer from overheating of the lipid suspension causing degradation. Sonication tips also tend to release titanium particles into the lipid suspension which must be removed by centrifugation prior to use. For these reasons, bath sonicators are the most widely used instrumentation for preparation of SUV. 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 T_c 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.

Extrusion*: Lipid extrusion is a technique in which a lipid suspension is forced through a polycarbonate filter with a defined pore size to yield particles having a diameter near the pore size of the filter used. Prior to extrusion through the final pore size, LMV suspensions are disrupted either by several freeze-thaw cycles or by prefiltering the suspension through a larger pore size (typically 0.2µm-1.0µm). This method helps prevent the membranes from fouling and improves the homogeneity of the size distribution of the final suspension. As with all procedures for downsizing LMV dispersions, the extrusion should be done at a temperature above the T_c of the lipid. Attempts to extrude below the T_c will be unsuccessful as the membrane has a tendency to foul with rigid membranes which cannot pass through the pores. Extrusion through filters with 100nm pores typically yields large, unilamellar vesicles (LUV) with a mean diameter of 120-140nm. Mean particle size also depends on lipid composition and is quite reproducible from batch to batch.



Removal of unencapsulated material:

The objective of many liposomal formulations is to encapsulate a compound of interest within the vesicle itself. These compounds can be either hydrophobic or hydrophilic and are often associated with fluorescent dyes, proteins, etc. In most cases in which it is desired to encapsulate a hydrophilic compound, the unecapsulated (free) compound needs to be removed. This is done utilizing size-exclusion gel chromatography. The dry gel should be hydrated 24 hours before use with a column buffer having the same osmotic potential as the aqueous phase of the liposomes. Once the gel is fully hydrated it is loaded into a suitably sized chromatography column. In order to have an optimal chromatography run, there must be no air bubbles in the gel once it is loaded into the column. The liposomes are loaded onto the gel and the column buffer is used to push the liposomes through the gel. The liposomes will elute from the column first and should be collected in fractions. A well run column may dilute the original lipid concentration up to 50%. All fractions containing liposomes can be combined and used for the research experiment.

The three illustrations shown on page 1 are excerpted from the book 'Liposomes in Gene Delivery' by Danilo D. Lasic, published 1997 by CRC Press LLC. Avanti thanks the publisher for kind permission to reproduce these drawings.

* Extrusion equipment available from Avanti. Product Number 610000

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