



Liposomes: A Review

Part I: Manufacturing Issues

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Liposomal formulations increasingly are being used in biopharmaceutical development projects. Good manufacturing processes must reproducibly fabricate lipid vesicles that are as uniform in size as possible, carry the drug in sufficient concentration, and remain stable for an acceptable shelf-life under varying temperatures.

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Phospholipid vesicles (liposomes) were first described decades ago by Bangham et al (1). It has been shown that phospholipids spontaneously form closed structures when they are hydrated in aqueous solutions. Such vesicles, composed of one or more phospholipid bilayer membranes, can carry aqueous or lipid drugs, depending on the nature of those drugs. Because lipids are amphipathic (both hydrophobic and hydrophilic) in aqueous media, their thermodynamic phase properties and self-assembling characteristics evoke entropically driven sequestration of their hydrophobic regions into spherical bilayers. Those layers are referred to as *lamellae*.

Liposomes vary in charge and in size depending on the method of preparation and the lipids used (the multilamellar vesicle [MLV] size range is 0.1–5.0 μm , the small unilamellar vesicle [SUV] size range is 0.02–0.05 μm , and the large unilamellar vesicle [LUV] size range varies from 0.06 μm and greater). They provide a number of important advantages (explored further in Part II of this article) over other dispersed systems, including high encapsulation of water-soluble drugs, lipid economy, and reproducible drug release rates. Liposomal preparations can replace some commercial products containing toxic solubilizing agents, thus providing useful alternative dosage forms for intravenous administration.

Pharmaceutical researchers use the tools of biophysics in evaluating liposomal dosage forms. Such combination activity is revealed in the increasing number of review articles and monographs incorporating both the physics and therapeutic applications of liposomes. In general, these publications include a definition of the liposomes studied, a description of how they are made and characterized, and a list of beneficial medical attributes for the liposomal drug delivery system. In this first of two parts, we focus on the former

two: production and benefits. Part II will examine drug delivery systems and their uses.

Liposome production

Options are numerous for combining phospholipids with an aqueous phase, but two major methods are used to make liposomal systems for drug delivery. The first is simple hydration of a lipid followed by high-intensity agitation using sonication or a high-shear impeller. Liposomes then are sized by filtration or extrusion. The second method is *emulsion*. Phospholipid first is dissolved in an organic solvent (such as methylene chloride) and then added under control to an aqueous medium with vigorous agitation. The organic solvent then is removed under reduced pressure. The resulting liposomal dispersion also is sized by filtration or extrusion. In general, the first method yields multiple-lamellae products, and the second method yields products with few lamellae.

Liposomes are difficult to produce. *Large* in this context usually means any structure >100 nm; thus LUVs are those above 100 nm in diameter that are bounded by a single bilayer membrane. Some authors have referred to 50–100 nm liposomes as “large,” and others have referred to those measuring less than 100 nm as “small.” Therefore, the definitions of *large* and *small* are very subjective, and that has complicated the understanding of liposome size.

Size distributions. Liposomes produced by the high-encapsulation injection process exhibit a broad size distribution in the range of 0.2–1.5 μm . Downsizing such liposomes generally results in a loss of encapsulated materials.

A technique that has gained widespread acceptance for producing liposomes of defined size and narrow size distribution involves the extrusion of a heterogeneous population of fairly large liposomes through polycarbonate membranes under moderate pressures. That simple technique can reduce a heterogeneous population to a somewhat homogeneous suspension of vesicles that exhibit a mean particle size near that of the pores through which they were extruded. That outcome may represent a rather broad distribution of vesi-

cle sizes, but compared with the original population (ranging from about 500 nm to several microns in size), it represents a considerable reduction of average particle size.

Incorporating drugs

To understand the performance characteristics of liposomal systems, it is important to understand the mechanisms of introducing drugs into liposomes. That process is achieved using one of three primary mechanisms: encapsulation, partitioning, and reverse loading.

Encapsulation. Useful for water-soluble drugs, the encapsulation is simple hydration of a lipid with an aqueous solution of drug. The formation of liposomes passively entraps dissolved drug in the interlamellar spaces, essentially encapsulating a small (captured) volume.

Partitioning. A drug substance that is soluble in organic solvents will go through partitioning. It is dissolved along with phospholipid(s) in a suitable organic solvent. That combination either is dried first or added directly to the aqueous phase, and residual solvent is removed under vacuum. The acyl chains of the phospholipids provide a solubilizing environment for the drug molecule, which will be located in the intrabilayer space.

Reverse loading. The reverse-loading mechanism uses the fact that certain drugs (such as weak acids) may exist in both charged and uncharged forms depending on the pH of their environment. Such drug molecules can be added to an aqueous phase in the uncharged state to permeate into liposomes through their lipid bilayers. Then the internal pH of the liposomes is adjusted to create a charge on the drug molecule. Once charged, the drug substance no longer is lipophilic enough to pass through the lipid bilayer and return to the external medium.

Use of liposomes to carry peptide and protein drugs and DNA vaccines involves simple, easily scaled technology that is capable of high-yield vaccine entrapment. A dehydration-rehydration technique applied for entrapping particulate antigens freeze-dries giant vesicles (4–5 μm in diameter) in the presence of spores. On rehydration and sucrose-gradient fractionation of the resultant suspension, 30% or more of the spores used will be associ-

ated with generated giant liposomes of similar mean size.

Characterization

In addition to concentrations of the drug and lipids in the vesicles, measurements of captured volume, size distribution, and *lamellarity* (the number of layers making up the shell of the bubble or vesicle) characterize lipid vesicles. The size of liposomes is considered an important factor in measuring liposome-complement interactions (2). In a study by Yamada et al., the release of carboxyfluorescein (CF) from liposomes was measured for three different diameters (800, 400, and 200 nm) by changing the liposome concentration from 1 to 1,000 nmol/mL (2). At a low liposome concentration range (1–10 nmol/mL), small liposomes (200 nm) released CF to a similar extent (about 35%) as medium (400 nm) and large (800 nm) liposomes.

The affinity (K_m) and capacity (λ_{max}) of a complement system to release liposomal encapsulated CF were estimated by kinetic analysis of the liposome-complement interaction. Surprisingly, no remarkable size dependency was found in the K_m or λ_{max} by liposome number, although parameters depended on the lipid concentrations. Those results indicate the possibility that the complement system does not discriminate according to liposome size. The study indicated that placental uptake and the transfer rate of liposomal CF were dependent on liposome size (3).

Mean vesicle size and size distribution.

These essential parameters describe the quality of liposome suspensions. They are important parameters for the physical properties and biological fate of liposomes and their entrapped substances in vivo. (Part II of this article will discuss those further.) A number of methods are used to determine size and size distribution, but one of the most commonly used methods is light-scattering analysis. A number of techniques are available to size liposomes based on that methodology. Light scattering is popular because of the

Table I: Dispersion sample — true statistical radius 15.9 nm, instrumental average 25.3 nm.

Percent of	Particle Size	
	15-nm	45-nm
Particles	97	3
Surface area	78	22
Total volume	54	46

Table II: Typical correlation between particle-size distribution and mean diameter.

Lot	Particle Size* %			Mean Particle Size*
	<0.05	0.05–0.45	>0.45	
A	1.5	98.4	0.1	0.132
B	1.6	96.4	0.1	0.132
C	1.1	98.9	0.0	0.128
D	1.6	97.9	0.4	0.148
E	3.6	96.4	0.0	0.114
X**	<10%	>80%	<10%	0.05–0.5

* in microns (μm)

** represents specifications

ease of operation and the speed by which scientists can obtain data. Newer instruments are based on laser light scattering.

If the liposomes to be analyzed are monodisperse, light-scattering analysis is the method of choice; unfortunately, most liposomal preparations are heterogeneous, and they require an accurate estimate of their size-frequency distributions. Light-scattering methods rely on algorithms to determine particle-size distributions, and the results obtained can be misleading. Some complex algorithms have been developed as an attempt to address that problem. However, such methods cannot distinguish between a large particle and a flocculated mass of smaller particles. Most important, it may be necessary to remove any micron-sized particles that are present in such masses before analysis.

Complications. The difficulty in interpreting data about particle size can be demonstrated with a simple example of a dispersion composed of 97% SUV with a radius of 15 nm and 3% MLV with a radius three times greater (45 nm). Table I lists the results. Thus, 3% of the particles constitute almost one-half the volume of the liposomes present. Similar problems of data analysis occur with other dispersion systems such as emulsions and suspensions.

Another difficulty in understanding size and size distribution is the range of distribution around the mean value. Single values for particle size in dispersion systems such as emulsions and suspensions reported in the literature are usually the average mean diameter. Each dispersion system has its own size distribution. Typically that size distribution is close to an average mean diameter. But two different dispersion systems with similar average mean diameters could have very similar size distribution.

A single value often is reported in the literature for mean diameter values that are different from the expected center of distribution. Table II provides an average mean diameter for a suspension in which the particle size distribution ranges from $<0.05\ \mu\text{m}$ to $0.5\ \mu\text{m}$, with a mean diameter $\sim 0.140\ \mu\text{m}$. Fewer than 4% of the particles are $<0.05\ \mu\text{m}$, and particles $>0.45\ \mu\text{m}$ are more than 0.4% of each lot reported. The specification set for both the lower and upper range is 10%. Set specifications indicate a wider spread than does the actual data, which shows a much tighter distribution in the middle and less than a few percent on the lower and upper ends. So the mean diameter of $0.140\ \mu\text{m}$ is far below the center of distribution, indicating that the particle-size distribution does not follow a normal distribution around the mean.

Theory into practice

A number of molecules can be quantitatively entrapped into the aqueous phase of liposomes by a dehydration–rehydration procedure in the absence of sonication, detergents, or organic solvents. “Empty” liposomes, preferably SUVs, are mixed with a solution of the drug destined for entrapment. The mixture is dehydrated by freeze-drying and the formed powder subsequently rehydrated under controlled conditions. That process forms multilamellar dehydration–rehydration vesicles (DRV) containing $\leq 80\%$ of the original solute. Significantly, microfluidization of the drug-containing DRV in the presence of nonentrapped solute generates smaller vesicles (down from about an 800 nm average mean diameter) with much of the originally entrapped solute still contained.

Low efficiency in encapsulating large DNA molecules into liposomes has been

an important technical obstacle to their use in gene delivery. That problem can be overcome by using cationic liposomes, which interact spontaneously with negatively charged nucleic acid polymers. Simply mixing positively charged liposomes with DNA incorporates 100% of the polynucleotide into a lipid–DNA complex. Furthermore, relatively homogeneous and physically stable suspensions can be obtained by carefully controlling the complexation conditions. Because of its convenience and efficacy, cationic lipid-mediated gene delivery technology has become a promising system for *in vivo* gene therapy. Clinical trials conducted on large-size lipid–DNA complexes have mostly shown a lack of adverse effects along with moderate expression in a relatively low fraction of cells — but no decisive clinical disadvantages.

Stability

The stability of a pharmaceutical product usually is defined as the capacity of its formulation to remain within defined limits during the shelf life of the product. Classical models from colloid science can be used to describe liposome stability. Colloidal systems are stabilized electrostatically, sterically, or electrosterically. In addition to normal colloids, self-assembling colloids can undergo fusion or phase change after aggregation. Liposome dispersions exhibit both physical and chemical stability characteristics. Generally, the physical characteristic describes the preservation of liposome structure, and the chemical characteristic refers to molecular structure.

Physically stable formulations preserve both liposome size distribution and the amount of the material encapsulated. Stability depends on the mechanical properties of the liposome membranes and on the thermodynamics and colloidal properties of the system.

Predicting the stability of liquid-dispersed systems is one of the most difficult problems faced by formulation chemists. Scientists often are asked to predict the shelf life of a product or choose between experimental formulations on the basis of mere estimates of how they will hold up over time. No standardized tests are available to determine physical stability, and quite often the type of stability being investigated is uncertain.

The priority for solving the stability problems of dispersion systems is to define clearly the type or types of stability of concern. Simply categorizing stability as either physical or chemical is insufficient. Understanding the factors that lead to stability problems can help determine which methods of testing are most likely to yield information applicable to estimating the product’s shelf life. Stability tests commonly stress a system to limits beyond those that the product will ever encounter.

Temperature studies. High-temperature testing ($>25\ ^\circ\text{C}$) is almost universally used for heterogeneous products. For liposomes, elevated temperatures may dramatically alter the nature of the interfacial film, especially if the phase-transition temperature is reached. If one expects the product to be exposed to a temperature of $45\ ^\circ\text{C}$ for extended periods (or even for short durations), studies at $45\text{--}50\ ^\circ\text{C}$ are quite justified. Studying a liposomal product at such temperatures determines how the product will hold up and whether any damage is reversible when the product is brought back to room temperature. If temperatures higher than the system will ever encounter are used — even in short-term heat–cool cycling — one risks irreversibly damaging the bilayers such that the membrane cannot heal when brought back to room temperature.

If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow at the expense of water. Liposomes then may press together against the ice crystals under great pressure. If crystals grow to sizes greater than the void spaces, instability is more likely. That phenomenon is well noted with a slower rate of cooling, causing formation of larger ice crystals, which leads to greater instability. Certain polymers are known to retard ice crystal growth.

Time studies. The zeta potential (ZP) and the dielectric constant (ϵ) of liposomes are measured to study the effects of *in vitro* aging. Aging studies show an increase in the ϵ and the ZP potential for liposomes at various storage temperatures. Both electrical parameters could be useful in studying structural alterations in liposomal vesicles and systems as a function of various conditions.

It is critical that stability-testing protocols for liposomal products be developed

case by case. Be certain that studies are performed using various types and sizes of containers. Under each test condition, the following data can be collected: visual and microscopic observations (flocculation, for example), particle size-profiles, rheological profiles, chemical stability, and extent of leakage.

Using liposomes

The ultimate goal of a liposome formulation is to deliver therapeutic molecules to their site of action in the patient's body. In Part II of this article, we will discuss the advantages of liposomal drug delivery systems (including targeted and sustained delivery) and their eventual fate in vivo as well as clearance from the body.

References

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FYI

Pharmacopeial education short course

United States Pharmacopeia (USP) has announced that its short course "Developing Standards for Pharmaceuticals and the Use of the *USP-NF*" will be offered through the University of Wisconsin (UW) Extension Services in Pharmacy 30 April–2 May 2002 at UW Madison.

The course is designed to help *USP-NF* users better understand pharmacopeial processes, tests, methods, and acceptance criteria and offers participants credits from the American Council on Pharmaceutical Education. Registration may be completed on-line at www.pharmacy.wisc.edu/esp/oncampus.html or by contacting Diana Lenahan at 301.816.8530.

For more information, contact USP, 12601 Twinbrook Pkwy., Rockville, MD 20852, tel. 800.822.8772, fax 301.816.8148, custsvc@usp.org, www.usp.org.