



High-Concentration Liposome and Micelle Suspensions: Measurement by Wave II Dynamic Light Scattering

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Application Note

SL-AN-07 Revision C

Provided By:

Microtrac, Inc.

Particle Size Measuring Instrumentation

APPLICATION NOTE

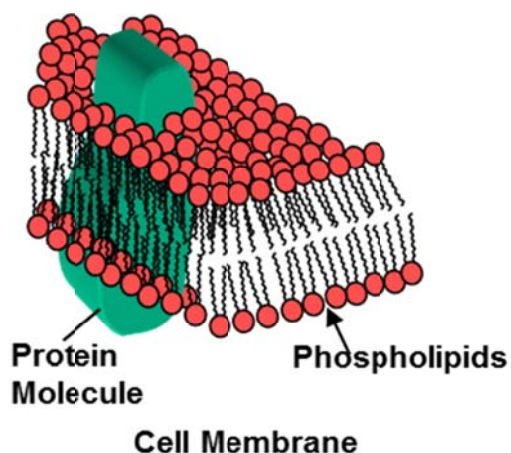


Solubility of Biological Compounds

Water disperses and dissolves many compounds. One such ionic species dissolvable in water is sodium chloride that by virtue of the dielectric properties of water can easily be separated into individually charged atoms, which can be hydrated (solvated). The dielectric constant (D) is inversely proportional to the distance between the ions while the attractive force is proportional to the ion charge. Thus water (D= 80) easily dissolves sodium chloride while the salt is insoluble in benzene (D=2.3). Another type of dispersal involves hydrogen bonding of water molecules to polar functional groups in the chemical structure of sugars and alcohols. Changes in the structure of water by use of surfactants or salts can be employed to “dissolve” proteins such as antibodies, collagen, some hormones, hemoglobin and myosin. Dispersal of the proteins generally depends upon the protein composition. Composition dictates which chemicals are necessary to reduce the forces of attraction that prevents solubility. A last type of dispersal includes biological chemicals. Lipids as a class, depending upon their structure, will dissolve in polar or non-polar organic solvents, but generally, this class of compounds is not soluble in water. Water insolubility allows lipids to enter special chemical bonding, which produces structures such as blood lipoproteins, cell membranes, micelles and liposomes. This paper describes lipoproteins and micelles with particular attention to particle size measurement of liposomes in either dilute or concentrated aqueous environments.

Cell Membranes

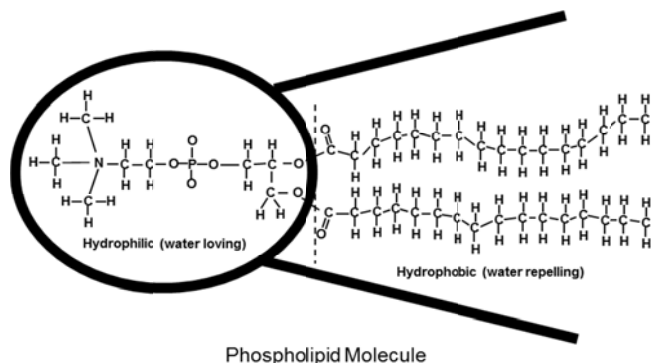
Biological tissues are comprised of cells whose contents perform the biochemical reactions that provide living organisms energy, tissue repair, respiration, immunity, etc. Each cell is composed of a number of different functional units called *organelles*. Each of these provides a locale for specific types of biological reactions. These organelles are “housed” in a structure termed the *cell membrane* which acts to maintain protection of the organelles, allow a highly controlled environment for chemical reactions, and provide a “gate” through which all substances must pass into or out-of the cell. There are a variety of mechanisms that provide the action but are too complex to describe here. The structure of the cell membrane has been studied for many years. From simple to complex models, the structure of any biological membrane is not completely defined and remains an area of great biotechnical interest but clearly contains lipids and proteins. These compounds interact to form the cell membrane and other structural components of cells. All compounds enter the cell by passing through the protein-lipid complex by one of several mechanisms e.g., diffusion, including mechanical means where the cell preferentially “opens” and interacts with other membranes to allow entrance of components (reverse pinocytosis)



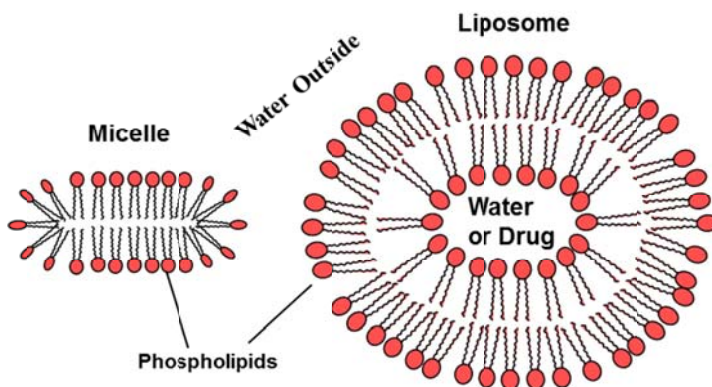
Liposomes and Micelles

One of the earliest studies of the interaction of lipids and proteins to form structures was performed by mixing particular polar lipids (contains water-loving portion in the molecule) with water. Such lipids may show little solubility in water or polar organic solvents, e.g. alcohols. A lipid popular for preparing liposomes or micelles is phosphatidylcholine, which can be classified as polar lipid. However, most polar lipids manifest dispersibility in both types of solvents because of their *amphipathic* (a molecule containing both highly non-polar portions and highly polar portions)

nature. Such a mixture will result in a two-phase system - water on the bottom and oil on top. If the mixture is agitated fiercely using a homogenizer or ultrasonic probe, it is possible to cause the lipids to form a mixture with water. In this case the molecule orients to allow the polar portion to interact with water while the non-polar portion orients away from water. The same orientation occurs when mixing surfactants (surface active agents) with water. Surfactant molecules are similar to phospholipids in that the molecule usually contains water-loving and water-repelling portions.



When mixed with water at very high concentrations, the molecules can form discrete units called **micelles**. Micelles have general structural features in common with membranes and liposomes in that special orientation of the molecules is required for formation. While micelles can be prepared using phospholipids, they do not contain water within the interior of the structure. These structures are used for preliminary modeling of complex biological structures, and prototype drug delivery systems. While the thickness of the structure may be 50 to 100 Å, the micelle diameter can range from nanometers to micrometers (1Å= 0.1 nm).



Liposomes are structures usually prepared using lipids such as phosphatidylcholine (C-16 is shown). These structures are often nearly spherical. As with micelles, the non-polar tails of the molecules are thermodynamically driven to orient inside the completed structure. The size of the completed structures typically ranges from 40 nanometers (transdermal drug delivery systems) to as large as several microns (cosmetics).

Drug Targeting and Delivery

Of special interest to the pharmaceutical arena is the use of the above concepts in the development of drugs for targeting and delivery. The lipid structures of the micelle can be produced from a variety of polar lipids including phosphatidylcholine and cholesterol. In **drug targeting**, the formation of the micelle also involves the steps necessary to encapsulate a drug species. This structure is called a **liposome** and was first described by Sessa and Weiss in the early 1970's who treated an immiscible mixture with ultrasonic energy. The energy produces optically clear "solutions" that contain closed (usually spherical) structures (vesicles or liposomes). Because of the chemical nature of the membrane, it is impermeable to nearly all other water-soluble components. Since the polar lipids and particularly **phospholipids** can be positively or negatively charged, liposomes may be made to vary

in characteristics. Liposomes having several layers of lipids (multi-lamellar) separated by water-drug solution have been produced to provide for time-release applications.

In one case the polar phosphatidyl portion of the molecule is oriented toward the outside of the liposome. This structure is similar to that of a blood cell and following injection can pass through the blood, kidneys and liver un-assailed and to a specific physiological site without premature release of the drug and potential side effects. In this way, a lower dosage is allowed to achieve the same biological activity used at a much higher oral dosage. In addition, the formulation is protected from the effects of the gastrointestinal tract where a variety of enzymes, inhibitors and pH effects can be encountered.

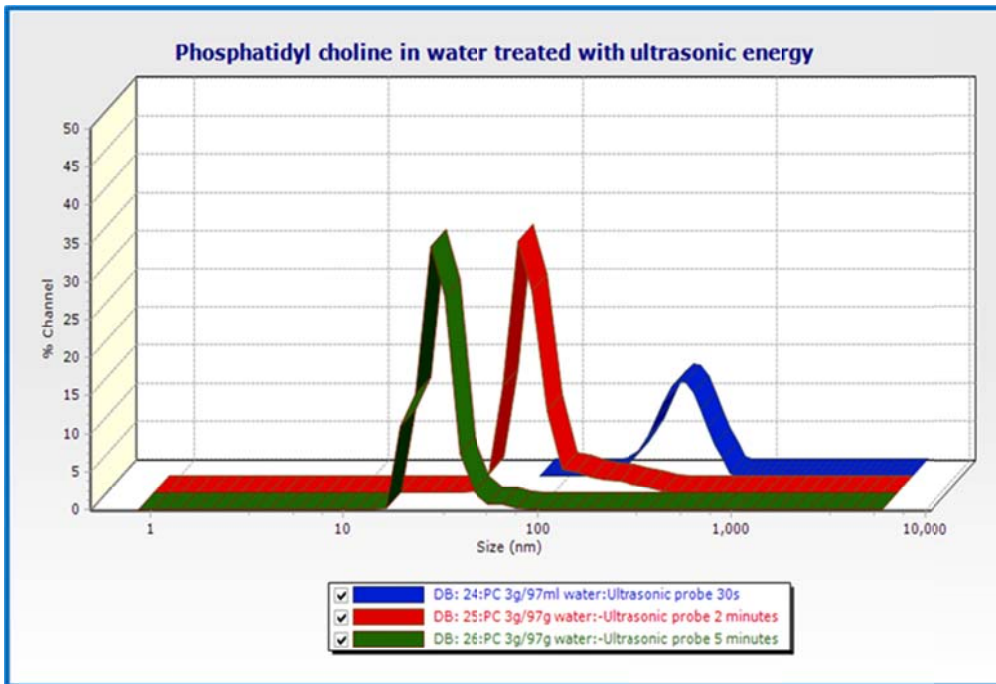
Selection of the type of lipids also determines the speed of cell absorption or delivery by either diffusion through the skin or cell membrane dissolution. Thus, as discussed above, the targeted cell membrane of tissue organs acts as a gate to this drug by selective interaction or reaction with the liposome shell. In addition, the liposome can be chemically composed in a manner to provide timed release of the drug to maintain its efficacy. An excellent example and discussion of this concept is presented by Nema, et. al who describes the **protection** of lactate dehydrogenase (an enzyme/protein) against **protease** digestion by encapsulation in **phosphatidyl choline**. The advantage of this approach is that the vesicles are non-toxic, non-immunogenic and biodegradable. Other means of protein protection such as complexation, polymer coating and administration of protease inhibitors do not necessarily offer these advantages. Liposomes also may be targeted very specifically to physiological sites by attaching monoclonal antibodies or specific receptors/antigens to the membrane and thus limiting recognition by other tissue membranes. Liposomes offer many other benefits such as, carrying both water soluble and oil soluble drugs in a single dose, solubilizing recalcitrant drugs, protein stabilization and controlled hydration.

Liposome Stability

In order to maintain liposome stability and shelf life, several factors must be considered. Items such as temperature, pressure, liposome composition, drug stability, ionic strength, and viscosity all may have stabilizing or destabilizing (manifested as unacceptable shelf-life) effects. One important method used to measure instability is *particle size*. Typically, liposomes must be smaller than 200 nm in order to pass through the blood system unobstructed. In addition, liposomes of this size are smaller than bacteria, mold spores, and fungi making the liposome easily separable from the microorganisms using a 200 nm filter. A possible future goal is to prepare liposomes of maximum size 100 nm to allow exclusion of most virus particles.

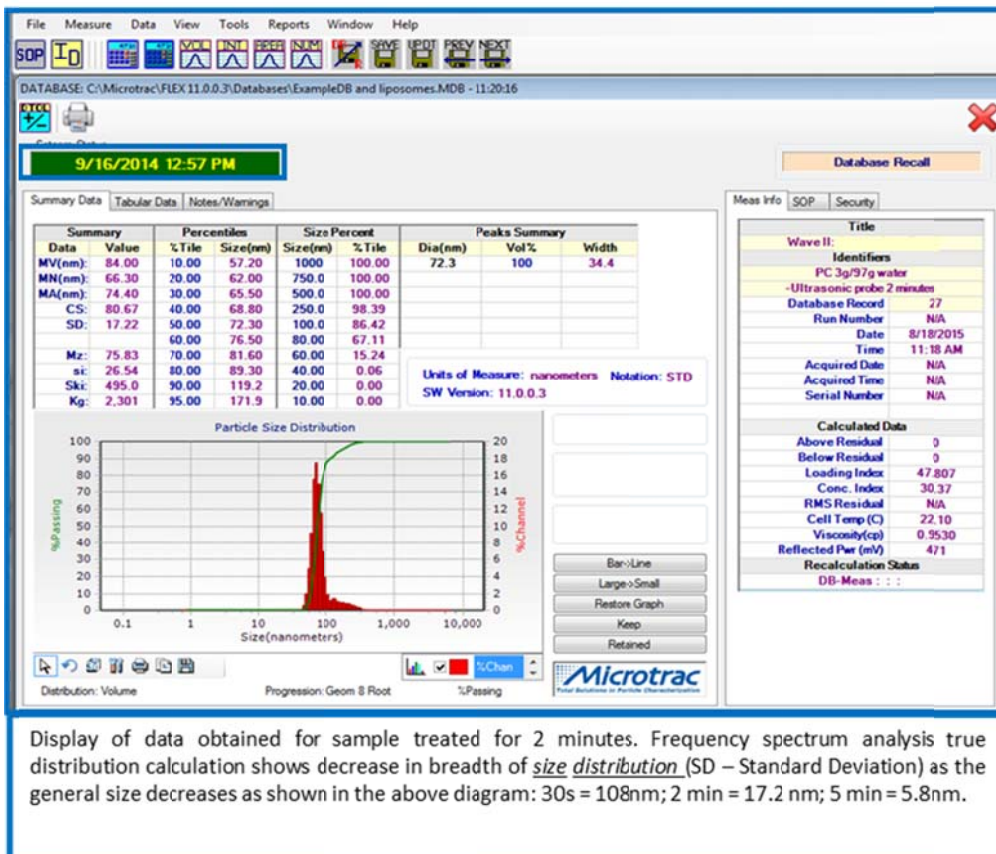
Size Measurement

As a direct result of the need to control, modify and stabilize the liposomes, size is a critical parameter to measure. Thus the growth of multi-lamellar structures, liposome agglomeration, membrane disruption, and microbiological contamination are of extreme importance and can be studied through particle size measurement. The major drawbacks to such measurement are: dilution requirement, measurement duration, and inadequate instrumentation capability. Dilution can result in misinterpreted data due to losses in particle-particle interactions or even destabilization of the preparation. Long measurement duration by chromatographic means, preclude rapid assessment of characteristics while chemicals used in the measurement may adversely and misleadingly affect the structure size. While Photon Correlation Spectroscopy (PCS) instrumentation can be employed to shorten measurement duration (5 to 30 minutes) and to measure ultrafine particles (3 to 6000 nm), the liposomes are subject to PCS dilution requirements and the attendant issues discussed above.



Three sizes of phosphatidylcholine (PC) liposomes prepared by mixing 3 grams PC with 97grams deionized water. Mixture was treated with an ultrasonic probe for 30 seconds, 2 minutes and 5 minutes resulting in three sizes of liposomes.

Note how the tail on the coarse end of the distribution disappears as ultrasonic treatment reaches 5 minutes



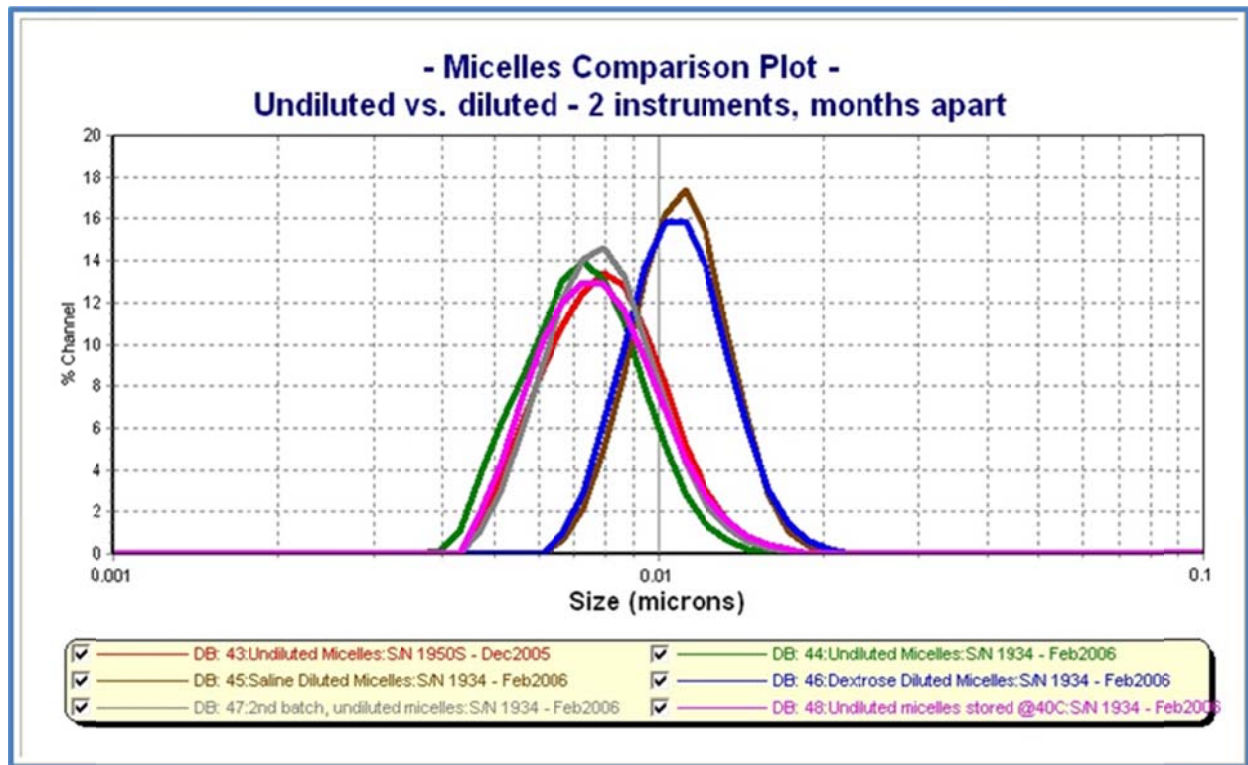
Display of data obtained for sample treated for 2 minutes. Frequency spectrum analysis true distribution calculation shows decrease in breadth of *size distribution* (SD – Standard Deviation) as the general size decreases as shown in the above diagram: 30s = 108nm; 2 min = 17.2 nm; 5 min = 5.8nm.

The Nanotracs employing the **“Controlled Reference Method”** surmounts these measurement issues. The measuring period is 30 to 100 seconds which reduces measurement time by as much as 30 to 360 minutes when compared to chromatographic, electrophoretic procedures or other DLS instruments. The measuring range of 0.8 to 6500 nm includes the size of very small micelles and proteins, to the larger liposomes. The measurement is independent of concentration thus allowing measurements at full strength (typically 0.01 to 40%) without use of other chemicals or dilution that can alter the liposome-emulsion components and cast doubt on test results.

The Wave II can withstand the production area environment so that production personnel can perform the measurement in proximity to the process. Lab time is freed to perform measurements requiring special training and skills. Since no special technique or training is required, the learning period is short while obtaining precision data.

Data from the production area are easily accessible and useable by others of various technical backgrounds including engineering and quality control. Wave II software provides easy save and recall of data records and is exportable by and to all modern electronic venues. Thus data can be presented by Wave II as hard copy reports, saved to CDs, exported to EXCEL, pasted into PowerPoint and Word, transmitted by HTML, ASCII, or customized to meet special report or presentation requirements.

Below is shown a stability experiment conducted using the Wave II on micelles. Dilution with saline and dextrose were shown to increase the size of micelles.





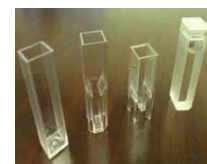
Wave II with Internal Cell

Wave II Capabilities

- Patented “Controlled Reference Method” for high signal and true particle size distribution by frequency analysis.
- Measures multimodal distributions without assumptions or “fitting”
- True background measurement eliminates need for high purity diluent or filtration operations.
- Withstands plant environment
- Full database management capability exportable using HTML, ASCII, pdf formats and to all popular spreadsheets and database managers
- High concentration measurement up to 40% solids
- Convenient, portable small size
- Simplicity of operation
- NO extensive sample preparation
- NO selection of special distribution models where operator decides on cumulants, NNLS, CONTIN or other complex choices.
- Compatible with most common organic solvents and aqueous solutions.
- Many models to choose from:
 - Internal cell (700 μ l) available in Teflon or stainless steel.
 - Cuvette cell (Wave Q) uses both disposable plastic and reusable glass cells.
 - External probe an excellent choice for:
 - Dip ‘N’ Run method (like making a pH measurement), or
 - OEM applications.



External Probe models measure directly into all types of containers or vessels



Wave II Q comes with a variety of polystyrene & glass cuvettes