FAQ

How are FormuMax's liposomes manufactured?

For liposomes with a drug loading battery of ammonium gradient, transmembrane ammonium gradient is generated by diafiltration (cartridges MWCO 100k-300K) against 8-10 volumes of 10% sucrose.
All liposome products are sterile filtered through 0.2um filters and fill in autoclaved vials in a BSC hood.

Typical products release assay include:

- Particle size, Half-width and polydispersity (Brookhaven ZETAPALS)
- Zeta potential (Brookhaven ZETAPALS)
- pH
- Total lipid concentration (The Stewart assay)
- Total or free drug concentration (UV, Fluorescence or HPLC)
- Free drug concentration (separation of free drug by SEC or filtration and assay the free drug concnetration)
- Encapsulation efficiency: calculated according to the free and total drug concentrations

Receiving and checking the liposome products

All our liposome products are well packaged and shipped overnight (or 2nd day) cold using a large box with a ¾inch Styrofoam inner box. We have test that the internal temperature inside the box stayed 10°C for two days when left inside a car in the summer in California. Carefully open the box and the inside should feel cool. Unpack the vials and check to make sure no vial broken. Visually exam the liposome suspension in each vial to make sure no visible particles/aggregates. The liposomes should be stored refrigerated at 2-8°C. On occasions, package could be in transportation for several days which have happened for international shippments due to custom clearance delays. Acidental exposure to ambient temperature for several days is usually not a problem for most products. The products should be carefully examined for turbidity and aggregations. But this situation should be avoided if possible.

How to transfer the Liposomes out of the vials?

The best way of transferring liposomes from the vials is to use a sterileneedle syringe. This way the remaining liposome suspension in the vial remains sealed and clean. You can also use a pipette with a sterile tip to transfer, but better to perform this in a

clean environment such as in a BSC hood.

Is the liposomes still good if it accidentally got frozen?

It depends. The liposome itself could actually survive upon freeze and thaw. So plain liposomes would most likely retain its original particle size and still good to use. The longer the lipid chains, the more stable the liposomes are. So pay extra attention for short chain DPPC liposomes and DOPC, POPC liposomes. Carefully check if the turbidity has changed and if aggregation has occurred. Check the particle size if you have a particle sizer.
However, for liposomes containing a drug loading battery, such as the ammonium sulfate liposomes, the ammonium gradient could have been collapsed and may have lost its ability to load drugs. Perform a test loading with a known drug is recommended.

Things to AVOID with liposomes?

Liposomes prepared from lipids with saturated hydrocarbon chains are actually very sturdy. But there are a few things one need to pay attention to.

- Do not mix with organic solvents, such as chloroform, methanol, ethanol, DMSO, ether and etc. chloroform will destroy the liposomes. Methanol or ethanol will soften or dissolve the membrane, depending on the amount used (up to 10w/v% is fine).
- Do not add surfactant, unless you purposely doing so to lyse the liposomes.
- Do not heat close to or above the lipid main phase transition temperature, unless doing a drug loading, which requires incubation above the phase transition temperature.

What are the plain liposomes for?

Plain liposomes are mostly used as placebo controls for *in vitro/in vivo* research. There are several other useful applications. Certain lipophilic small molecules can be loaded into liposomes by simple incubation of the two parts at an elevated temperature. In this case the molecules are actually incorporated into the membranes, instead of encapsulated inside the liposomes.

What are the ammonium gradient liposomes and what they are used for?

Ammonium gradient liposomes are liposomes with ammonium sulfate encapsulated inside. The outside ammonium sulfate has been replaced with a biocompatible solution, such as 10% sucrose or saline.

Ammonium liposomes are the best liposome system so far for actively loading weakly basic. It is so-called active loading, because the drug is driven into the pre-formed ammonium liposomes by simple incubation at a temperature above the main phase transition.

See reference:

Emanuel N, Kedar E, Bolotin EM, Smorodinsky NI, Barenholz Y. (1996) Preparation and characterization of doxorubicin-loaded sterically stabilized immunoliposomes (Link)

What types of compounds can be loaded into the ammonium liposomes?

The ammonium liposomes is used for active loading of small molecules that are weakly basic and lipophilic. Note not all basic comounds could be loaded this way, depending on the physiochemical properties. Examples of compounds that have been successfully loading into ammonium liposomes include doxorubicin, vincristine, topotecan, and etc. These compounds typically have an amino/amine group with pKa values in the range of 7-9. They have limited water solubility and most importantly with Log P values >0, meaning being lipophilic when deprotonated.

When to use PEGylated liposomes and when to use non-PEGylated liposomes?

The potential benefits of encapsulating a drug inside liposomes are to increase it solubility of insoluble compounds and/or to improve the PK/PD profiles. There is no need to use PEGylated liposomes, if drug solubility enhancement is the only goal. One can test if non-PEGylated liposomes prepared from saturated PC or from unsaturated PC, such as DOPC or POPC could provide satisfactory results. However, for increasing the PK/PD of a encapsulated compound, things are a little bit more complicated and it all depends on how well the drug can be retained with the liposomes. The carrier itself has an intrinsic blood circulation profile. $t_{1/2}$ =20-30 hr in rodents for PEGylated liposomes and $t_{1/2}$ =8 -15 hrs for non-PEGylated liposomes depending on the particle size. So when to use PEGylated liposome and when to use non-PEGylated? It depends on how well the drug stays with the carriers during blood circulation. Non-PEGylated liposomes would be sufficient, if the drug leaks out off the liposomes fast relative to the circulation of the carrier. Take drug A as an example, a $t_{1/2}$ of 5 hr was obtained when encapsulated inside PEGylated DSPC liposomes. In this case, one would expect a similar $t_{1/2}$ could be obtained with the non-PEGylated DSPC liposomes. On the other end of the scale, a drug that is very stably retained inside liposomes, such doxorubicin HCl ($t_{1/2}$ 20-30 hrs in rodents), it only makes sense to use PEGylated liposomes in order to achieve the best PK/PD possible with liposomes.

How to choose liposomes with the appropriate lipid chain length?

There are various factors when choosing a lipid chain length. The primary function of the lipid bilayers is to act as a drug permeation barrier. The longer the lipid chains the tighter the bilayers. The most commonly used liposomes are DSPC or HSPC based. However, when the drug retention still falls short, longer chain lipid could be considered. We have PEGylated and non-PEGylated 20:0PC, 22:0PC and even 24:0PC liposomes.

Why DSPC or HSPC liposomes are the most commonly used Lipids?

You may ask why don't we just use 24:0PC liposomes for everything, since it offers the potential for the slowest drug release? Well, the main reason of choosing DSPC (or HSPC) first is that it has the most desirable main phase transition temperature (T_m) of about 55°C. So the liposome membrane is deep in the gel phase (where lipid chains are tightly packed and drug release is slow) when injected into the body. On the other hand, liposomes need to be manufactured (hydration and extrusion) a few (5-10) deg C higher than the T_m of the lipid. This is not an issue for DSPC liposomes, but it becomes more and more challenging for longer chain lipids, because certain equipmentsand could not handle the high temperatures. See Table for the T_m values and required hydration/extrusion temperatures for preparing PC liposomes.

Lipids	Tm (deg C)	Hydration/Extrusion Temperature (deg C)
DPPC	41	46-51
DSPC/HSPC	55/54	60-65
20:0PC	66	71-76
22:0PC	75	80-85
24:0PC	80	85-90

What is the difference between HSPC and DSPC?

HSPC stands for fully hydrogenated soy PC. HSPC contains primarily 18:0PC (approx. 85% DSPC and 15 % DPPC). Its T_m is about 54 deg C. HSPC is used in commercial products like Doxil® and DSPC is used in DaunoXome®.Our liposomes for R&D are typically manufactured by the ethanol hydration and extrusion approach. Briefly, lipid mixtures are first dissolved in ethanol and then hydrated at a temperature above the lipid main phase transition with a hydration solution (i.e. HEPES buffered 10% sucrose solution or an ammonium sulfate solutions). Liposome down-sizing is achieved by extrusion through polycarbonate membranes using an extruder. The final particle sizes are typically 90-120nm as measured by a DLS particle sizer.