

Description**BACKGROUND OF THE INVENTION****1. Field of the Invention**

This invention relates to liposomes and in particular to dehydrated liposomes which can be stored for extended periods of time and then rehydrated when and where they are to be used.

2. Description of the Prior Art

As is well known in the art, liposomes are closed vesicles having at least one lipid bilayer membrane surrounding an aqueous core. One of the primary uses for liposomes is as carriers for a variety of materials, such as, drugs, cosmetics, diagnostic reagents, bioactive compounds, and the like.

In connection with each of these uses, it is important to be able to store liposomes for long periods of time without substantial leakage from the liposomes of the selected materials they are carrying. More particularly, so as to be useful in commercial settings, liposome preparations must have long enough shelf-lives to allow them to be easily manufactured, shipped, and stored by intermediate and ultimate users under a variety of temperature conditions.

With particular regard to the drug industry, it is also important to be able to provide drug manufacturers with unloaded liposomes which the manufacturers can subsequently load in their own plants with their own drugs. Such a two step or two factory approach (i.e., manufacturing unloaded liposomes in a first plant and then filling them in a second plant) would allow drug manufacturers to purchase a defined commodity, i.e., unloaded liposomes, from suppliers and then use that commodity as an off-the-shelf component of their final product.

As drug manufacturers currently operate their businesses, they strongly prefer to buy defined commodities from suppliers and then assemble the final product in their own plants. In this way, they can personally control the quality of the finished products. Usage of liposome technology by the drug industry would be greatly enhanced if liposomes could also be provided to manufacturers as a defined commodity.

To date, liposome preparations have generally had relatively short shelf-lives. Moreover, there has been no known way to prepare liposomes at one point in time and then fill them with selected materials at a much later point in time. The present invention makes up for these existing shortcomings in the current state of the art.

SUMMARY OF THE INVENTION

In view of the above state of the art, it is an object of the present invention to provide liposome preparations which can be stored for extended periods of time without substantial leakage from the liposomes of internally encapsulated materials.

It is a further object of the present invention to provide liposome preparations which can be dehydrated, stored for extended periods of time while dehydrated, and then rehydrated when and where they are to be used, without losing a substantial portion of their contents during the dehydration, storage and rehydration processes.

It is an additional object of the present invention to provide liposome preparations which can be dehydrated, stored for extended periods of time while dehydrated, rehydrated, and then filled with selected materials.

To achieve these and other objects, the invention, in accordance with one of its aspects, provides a method for dehydrating liposomes as claimed in claims 1 to 7 and liposome preparations which have been dehydrated in the presence of one or more protective sugars as claimed in claims 8 to 12. In certain preferred embodiments of the invention, the liposomes are dehydrated with the one or more sugars being present at both the inside and outside surfaces of the liposome membranes. In other preferred embodiments, the sugars are selected from the group consisting of trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose.

The dehydration is done under vacuum and can take place either with or without prior freezing of the liposome preparation.

In accordance with other aspects of the invention, delayed loading of preformed liposomes is achieved by creating a concentration gradient across the liposome membranes of one or more charged species which, under suitable conditions, are capable of passing across those membranes. This concentration

gradient is used to load selected charged materials, e.g., drugs, into the liposomes through the creation of a transmembrane potential.

It has been found that liposomes having a concentration gradient across their membranes can be dehydrated in the presence of one or more sugars, as described above and in more detail below, stored in their dehydrated condition, subsequently rehydrated, and the concentration gradient then used to create a transmembrane potential which will load charged materials into the liposomes. Alternatively, the concentration gradient can be created after the liposomes have been dehydrated, stored, and rehydrated.

The attainment of the foregoing and other objects and advantages of the present invention is described fully below in connection with the description of the preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the retention of $^{22}\text{Na}^+$ by dehydrated/rehydrated vesicles as a function of trehalose concentration. Large unilamellar vesicles were dried without prior freezing (open circles), or after freezing in liquid nitrogen (open squares).

Figure 2 shows freeze-fracture electron micrographs of vesicles before (Fig. 2a -- Control) and after (Fig. 2b -- Freeze Dried) dehydration/rehydration with prior freezing. Egg phosphatidylcholine vesicles were prepared by extruding large multilamellar vesicles through a 100 nm polycarbonate filter. The vesicles were dehydrated in the presence of 250 mM trehalose.

Figure 3 shows freeze-fracture electron micrographs of egg phosphatidylcholine vesicles before and after dehydration. The vesicles were prepared by extruding large multilamellar vesicles through a 100 nm polycarbonate filter. Fig. 3a shows the vesicles prior to dehydration. Fig. 3b shows the much larger structures obtained by dehydrating and then rehydrating the vesicles without the use of trehalose. Fig. 3c and Fig. 3d show the vesicles after dehydration and rehydration in the presence of 50 mM and 125 mM trehalose, respectively. The diameter of the large liposome in Fig. 3b is approximately 900 nm and the arrows in the upper right hand corners of the figures indicate the direction of shadowing.

Figure 4 shows the retention of ^3H -inulin as a function of trehalose concentration. Large unilamellar vesicles containing entrapped ^3H -inulin were dried under high vacuum without prior freezing.

Figure 5 shows the influence of sodium chloride concentration on the amount of $^{22}\text{Na}^+$ retained by dehydrated/rehydrated vesicles. The vesicles were dried in the presence of 250 mM trehalose under high vacuum for 24 hours.

Figure 6 shows the transmembrane potentials generated by a pH gradient for control vesicles (squares) and dehydrated/rehydrated vesicles (circles). Vesicles with a pre-existing proton gradient were maintained at 4°C for 24 hours (control) or dehydrated in the presence of 250 mM trehalose under high vacuum for the same period of time. The potential observed in the vesicles upon rehydration was determined in the absence of CCCP (open circles and squares), or with 20 μM CCCP present (solid circles and squares), using the probe ^3H -tetraphenylphosphonium bromide. The transmembrane potentials observed in vesicles without a pH gradient in the presence and absence of CCCP is shown by the solid and open triangles, respectively.

Figure 7 shows the transmembrane potentials generated by a Na^+/K^+ chemical gradient for control vesicles (squares) and dehydrated/rehydrated vesicles (circles). Vesicles with a pre-existing Na^+/K^+ gradient were maintained at 4°C for 24 hours (control) or dehydrated in the presence of 250 mM trehalose under high vacuum for the same period of time. The potential observed in the vesicles upon rehydration was determined in the absence of valinomycin (solid circles and squares), or with 0.5 $\mu\text{g}/\mu\text{mole}$ phospholipid valinomycin present (open circles and squares), using the probe ^3H -tetraphenylphosphonium bromide. The transmembrane potentials observed in vesicles having potassium glutamate on both sides of the membrane in the presence and absence of valinomycin is shown by the open and solid triangles, respectively.

Figure 8 illustrates the use of a transmembrane potential to load adriamycin to previously dried vesicles. Vesicles with a pre-existing Na^+/K^+ gradient were dehydrated for 24 hours in the presence of 250 mM trehalose. Following rehydration the ability of the vesicles to accumulate adriamycin in the presence (open circles), or absence (solid circles) of valinomycin (0.5 $\mu\text{g}/\mu\text{mole}$ phospholipid) was measured. Control vesicles maintained at 4°C for the same period were also tested in the presence (open squares) or absence (solid squares) of valinomycin.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above, the present invention relates to liposomes which can be subjected to long-term storage without substantial loss of their internal contents. The liposomes are stored in a dehydrated state,

and the dehydration is performed in the presence of one or more protective sugars.

The liposomes which are to be dehydrated can have a variety of compositions and internal contents, and are in the form of large unilamellar vesicles.

The liposomes can be prepared by any of the techniques now known.

Techniques used for producing large unilamellar liposomes (LUVs), such as, reverse-phase evaporation, infusion procedures, and detergent dilution, can be used to produce the liposomes. A review of these and other methods for producing liposomes can be found in the text *Liposomes*, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1. See also Szoka, Jr., et al., (1980) *Ann. Rev. Biophys. Bioengr.*, 9:467. A particularly preferred method for preparing LUVs is described in commonly assigned and copending WO 86/00238 and entitled "Extrusion Technique for Producing Unilamellar Vesicles,".

As other alternatives, the liposomes can be produced in accordance with the procedures described in US-A-4 522 803, US-A-4 588 578 US-A-4 721 612, referred to above, or in accordance with the freeze and thaw procedures described in U.S. Patent Application Serial No. 752,423, also referred to above. Also, rather than using liposomes per se, other lipid-containing particles, such as those described in US-A-4 610 868, referred to above, can be used in the practice of the present invention. Furthermore, if desired, the liposomes or lipid-containing particles which are to be dehydrated can be given a more uniform size distribution by subjecting them to the process of commonly assigned and copending WO 86/00238, and entitled "Liposomes Having Defined Size Distributions,".

The liposomes are preferably dehydrated using standard freeze-drying equipment or equivalent apparatus, that is, they are preferably dehydrated under reduced pressure. If desired, the liposomes and their surrounding medium can be frozen in liquid nitrogen before being dehydrated. Alternatively, and quite surprisingly, the liposomes can also be dehydrated without prior freezing, by simply being placed under reduced pressure. Dehydration without prior freezing takes longer than dehydration with prior freezing, but the overall process is gentler without the freezing step, and thus there is in general less damage to the liposomes and a corresponding smaller loss of the internal contents of the liposomes. For example, dehydration without prior freezing at room temperature and at a reduced pressure provided by a vacuum pump capable of producing a pressure on the order of 1 mm of mercury can take between approximately 24 and 36 hours, while dehydration with prior freezing under the same conditions can take between approximately 12 and 24 hours.

So that the liposomes will survive the dehydration process without losing a substantial portion of their internal contents, it is important that one or more protective sugars be available to interact with the liposome membranes and keep them intact as the water in the system is removed. A variety of sugars can be used, including such sugars as trehalose, maltose, sucrose, glucose, lactose, and dextran. In general, disaccharide sugars have been found to work better than monosaccharide sugars, with the disaccharide sugars trehalose and sucrose being most effective. Other more complicated sugars can also be used. For example, aminoglycosides, including streptomycin and dihydrostreptomycin, have been found to protect liposomes during dehydration.

The one or more sugars are included as part of either the internal or external media of the liposomes. Most preferably, the sugars are included in both the internal and external media so that they can interact with both the inside and outside surfaces of the liposomes' membranes. Inclusion in the internal medium is accomplished by adding the sugar or sugars to the solute which the liposomes are to encapsulate. Since in most cases this solute also forms the bathing medium for the finished liposomes, inclusion of the sugars in the solute also makes them part of the external medium. Of course, if an external medium other than the original solute is used, e.g., to create a transmembrane potential (see below), the new external medium should also include one or more of the protective sugars.

The amount of sugar to be used depends on the type of sugar used and the characteristics of the liposomes to be protected. As illustrated by Examples 1-5, below, persons skilled in the art can test various sugar types and concentrations to determine which combination works best for a particular liposome preparation. In general, sugar concentrations on the order of 100 mM and above have been found necessary to achieve the highest levels of protection. In terms of moles of membrane phospholipid, millimolar levels on the order of 100 mM correspond to approximately 5 moles of sugar per mole of phospholipid.

Once the liposomes have been dehydrated, they can be stored for extended periods of time until they are to be used. The appropriate temperature for storage will depend on the make up of the liposomes and the temperature sensitivity of the encapsulated materials. For example, as is well known in the art, various drug preparations are heat labile, and thus dehydrated liposomes containing such drugs should be stored under refrigerated conditions so that the drugs do not lose their potency. Also, for such drugs, the dehydration process is preferably carried out at reduced temperatures, rather than at room temperature.

When the dehydrated liposomes are to be used, rehydration is accomplished by simply adding an aqueous solution, e.g., distilled water, to the liposomes and allowing them to rehydrate. The liposomes can be resuspended into the aqueous solution by gentle swirling of the solution. The rehydration can be performed at room temperature or at other temperatures appropriate to the composition of the liposomes and their internal contents.

As discussed above, for certain applications, e.g., drug administration, it is desirable to be able to separate the process of loading liposomes from the process of preparing them. This can be accomplished by creating a transmembrane potential across the membranes of preformed liposomes and using that transmembrane potential to load charged materials, e.g., charged drugs, into the liposomes. The transmembrane potential is generated by creating a concentration gradient for one or more charged species (e.g., K^+ and/or H^+) across the liposome membranes. The concentration gradient is created by producing liposomes having different internal and external media, i.e., internal and external media having different concentrations of the one or more charged species.

Specifically, liposomes are prepared which encapsulate a first medium having a first concentration of the one or more charged species. For a typical liposome preparation technique (see discussion above), this first medium will surround the liposomes as they are formed, and thus the liposomes' original external medium will have the same composition as the first medium. To create the concentration gradient, the original external medium is replaced by a new external medium having a different concentration of the one or more charged species. The replacement of the external medium can be accomplished by various techniques, such as, by passing the liposome preparation through a gel filtration column, e.g., a Sephadex column, which has been equilibrated with the new medium, or by centrifugation, dialysis, or related techniques.

Depending upon the permeability of the liposome membranes, the full transmembrane potential corresponding to the concentration gradient will either form spontaneously or a permeability enhancing agent, e.g., an ionophore, such as, valinomycin, may have to be added to the bathing medium. (Note that, if desired, the permeability enhancing agent can be removed from the preparation after loading has been completed using chromatography or other techniques). In either case, a transmembrane potential having a magnitude defined by the Nernst equation will appear across the liposomes' membranes. This transmembrane potential will cause charged materials, e.g., charged drugs, to be loaded into the liposomes. Specifically, the transmembrane potential will cause those materials whose charge is opposite to the internal potential of the liposomes (outside ground) to accumulate within the liposomes. Thus, by adding to the external medium the material one wants to load and by choosing the concentration gradient and thus the transmembrane potential to have the appropriate orientation, loading of the liposomes can be accomplished as a separate operation from the creation of the liposomes.

The combination of transmembrane potential loading and liposome dehydration allows for great flexibility in the overall procedure for producing the finished, loaded liposomes. For example, liposomes having the same internal and external media, i.e., no transmembrane potentials, can be prepared, dehydrated, stored, rehydrated, and then the external medium can be replaced with a new medium having a composition which will generate transmembrane potentials, and the transmembrane potentials used to load the liposomes. Alternatively, liposomes having internal and external media which will produce transmembrane potentials can be prepared, dehydrated, stored, rehydrated, and then loaded using the transmembrane potentials.

In either case, when in their dehydrated state, the unloaded liposomes can be stored, shipped and otherwise easily handled. In particular, the dehydrated, unloaded liposomes are exactly the type of defined commodity which drug manufacturers prefer to purchase and thus satisfy the long felt need for a liposome product of this type (see discussion above).

A particularly important application of these transmembrane potential loading and/or liposome dehydration procedures is in the area of the administration of antineoplastic agents, such as, adriamycin (see Examples 1 and 6, below). A further discussion of these applications can be found in copending and commonly assigned WO 86/01102 and entitled "Encapsulation of Antineoplastic Agents in Liposomes".

The present invention will be more fully described by the following examples. The materials and methods which are common to the various examples are as follows.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC) was isolated employing standard procedures (see, for example, Singleton, et al., (1965) Journal of the American Oil Chemical Society, 42:53) and was more than 99% pure as determined by TLC. Trehalose, maltose, sucrose and glucose were obtained from the Sigma Chemical Company (St. Louis, Mo.), while lactose was purchased from Fisher Scientific Company (Fairlawn, N.J.). $^{22}\text{Na}^+$, ^3H -inulin, ^3H -tetraphenylphosphonium bromide and ^3H - H_2O were obtained from New England Nuclear (Lachine, Quebec). Adriamycin was obtained from Adria Laboratory (Mississauga, Ontario).

Vesicle Preparation

Vesicles were prepared using the extrusion techniques described in WO 86/00238, referred to above. A complete description of the techniques used appears in this application. Vesicles prepared by these techniques will be referred to herein as ETVs, i.e., Extrusion Technique Vesicles.

Briefly, 80 umoles of egg phosphatidylcholine were hydrated with 2 ml of 150 mM NaCl, 20 mM HEPES (pH 7.4) containing the indicated concentration of trehalose or other sugars. In those cases where the amount of residual water present after dehydration was determined, ^3H -water (30 uCi) was added to the HEPES buffer/sugar solution. $^{22}\text{Na}^+$ (5 uCi) or ^3H -inulin (5 uCi; specific activity 409 mCi/g) were added to the dry lipid prior to hydration.

The mixture was dispersed by vortexing and then passed ten times through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Inc., Pleasanton, CA) using a pressure of 250 psi. In those cases where a freeze-thaw procedure was utilized, the vesicles were prepared as above, freeze-thawed once using liquid nitrogen, and then again repeatedly passed through the stacked filters.

Unencapsulated $^{22}\text{Na}^+$ or ^3H -inulin was removed by passing the vesicles through a column (1.4 x 10 cm) of either Sephadex G-50 (fine) for removal of $^{22}\text{Na}^+$ or Ultragel AcA 34 for removal of ^3H -inulin. This procedure generally diluted the phospholipid content of the sample by approximately 50% to give a concentration of about 20 umoles phospholipid per milliliter.

Dehydration.

Samples (1 ml) were dried in 10 ml Kimex tubes at room temperature under high vacuum using a Virtis Freeze Drier (Gardiner, N.Y.). In some cases, the samples were frozen in liquid nitrogen prior to dehydration. In either case, the reduced pressure dehydration process was carried out for approximately 24 hours.

Rehydration

Following dehydration and storage for periods ranging from 1 to 7 days, the samples were rehydrated with distilled water (900 ul) and the vesicles dispersed by gentle vortexing. The amount of entrapped $^{22}\text{Na}^+$, ^3H -inulin or adriamycin remaining within the vesicles was measured using the techniques described below (see "Assays") after passage of 100 ul aliquots of the vesicle suspension over columns (1 ml) of Sephadex G-50 (fine) or Ultragel AcA 34, equilibrated with the same solution in which the vesicles were suspended, to remove any untrapped material (see WO 86/00238).

Since the columns tend to trap a small percentage of the liposomes applied thereto, the values reported below for the amounts of encapsulated material retained after the dehydration/rehydration process are somewhat lower than the levels actually achieved by the procedures of the present invention.

Freeze Fracture Electron Microscopy

Samples for freeze-fracture contained 25% glycerol and were fractured and replicated following the procedures described in Madden, T.D., Hope, M.J. and Cullis, P.R. (1983) Biochemistry 22, 1970-1974, using a Balzers freeze-fracture apparatus. Replicas were visualized on a Phillips 400 electron microscope.

Quasi-Elastic Light Scattering Measurements

Vesicles were sized employing a Nicomp 200 Laser Particle Sizer (Nicomp Instrument, Goleta, CA) operating at 632.8 nm and 5 mW.

Assays

Phospholipids were quantified by determination of inorganic phosphorus as described by Chen, et al., (1956) Anal. Chem. 28:1756. Adriamycin uptake was measured following solubilization of vesicles in 0.5% Triton X-100 from its absorbance at 480 nm. ^3H -inulin, ^3H - H_2O and ^3H -tetraphenylphosphonium were counted in a Phillips PW 4700 liquid scintillation counter, while $^{22}\text{Na}^+$ was quantified by gamma counting on a Beckman Gamma 800.

Example 1Dehydration of Liposomes Using the Protective Sugar Trehalose

This example illustrates the ability of the sugar trehalose to protect liposomes from substantial loss of their internal contents during dehydration and subsequent rehydration. Experiments demonstrating high retention levels for $^{22}\text{Na}^+$, ^3H -inulin, and adriamycin were performed. The results are shown in Figures 1-4 and Table 1.

In particular, egg phosphatidylcholine ETVs were prepared as described above using solute solutions containing $^{22}\text{Na}^+$ and various concentrations of trehalose. The ETVs were dehydrated with or without prior freezing in liquid nitrogen, rehydrated and assayed as described above. The results are shown in Figure 1.

As shown in that figure, the amount of $^{22}\text{Na}^+$ retained in the vesicles after rehydration depends on the trehalose concentration, with up to 90% of the sodium being retained at the highest concentration of trehalose tested (500 mM). As also shown in that figure, vesicles dehydrated without prior freezing retain more of their contents than those frozen in liquid nitrogen. That is, dehydration without freezing is overall a gentler process than dehydration with freezing.

The ability of trehalose to protect liposomes during dehydration/rehydration is further illustrated by the freeze-fracture electron micrographs of Figure 2 which shows liposomes before (Fig. 2a -- Control) and after (Fig. 2b -- Freeze Dried) dehydration/rehydration with prior freezing. The trehalose concentration in this case was 250 mM. As can be seen in this figure, the appearance of the liposome population is essentially unchanged by the dehydration/rehydration process, that is, the trehalose successfully protects the liposomes during this process.

Experiments performed without any trehalose in the solute solution gave a milky suspension upon rehydration in contrast to the opalescent appearance of the original sample. In addition, recovery of vesicles from the 1 ml Sephadex columns used to strip unencapsulated $^{22}\text{Na}^+$ was low (less than 10%), indicating that the liposomes had fused to form larger structures.

Figure 3 shows the effects of varying the trehalose concentration. Fig. 3a shows the liposomes prior to drying, while Fig. 3b shows them after drying and rehydration in the absence of trehalose. As is evident from these figures, the original vesicles are small and uniform in size, while the dehydrated/rehydrated vesicles are in general much larger.

Fig. 3c and Fig. 3d show liposomes which have been dried and rehydrated in the presence of 50 mM and 125 mM trehalose, respectively. As shown by these figures, vesicles dried in the presence of 50 mM trehalose and then rehydrated are generally the same size as prior to dehydration, but a small fraction of larger structures are also observed. At trehalose concentrations of 125 mM or greater, there is no discernible structural difference between vesicles before and after dehydration and rehydration.

To verify that vesicles dehydrated in the presence of trehalose retain their contents and do not simply re-encapsulate label upon rehydration, vesicles were prepared in 250 mM trehalose, and $^{22}\text{Na}^+$ was then added to the external medium. Following dehydration and rehydration, aliquots of the suspension were passed down 1 ml Sephadex columns as described in Materials and Methods above. Of the available $^{22}\text{Na}^+$, less than 0.02% was sequestered by the rehydrated vesicles, confirming that they do not encapsulate solute in the external medium upon rehydration.

The ability of dehydrated ETVs to retain inulin (molecular weight 5000) is shown as a function of trehalose concentration in Figure 4. A comparison of that figure with Figure 1, reveals that for the same trehalose concentration, more of the high molecular weight inulin is retained than the low molecular weight sodium. However, at the higher trehalose concentrations, the difference is quite small, suggesting that the

small amount of each label lost may be the result of vesicle rupture rather than permeability changes.

The ability of ETVs to retain the antitumor drug adriamycin when dehydrated in the presence of trehalose is shown in Table 1. The data presented in this table was obtained as follows: Egg phosphatidylcholine ETVs were prepared as described above using a solute solution (169 mM KGlu, 20 mM HEPES (pH 7.4), 40 μ mol lipid/ml) containing 250 mM trehalose. Subsequently, the external potassium buffer was exchanged for a sodium buffer (150 mM NaCl, 20 mM HEPES (pH 7.4), 250 mM trehalose). Adriamycin (200 nmol/ μ mol lipid) was added, along with valinomycin (0.5 μ g/ μ mol lipid) to induce a membrane potential. After a 2 hour incubation, unencapsulated adriamycin was removed by passing the vesicles through a column of Sephadex G-50 equilibrated with the trehalose-containing sodium buffer described above. The ETVs were dehydrated for 24 hours without prior freezing and then rehydrated as described above.

The amounts of entrapped adriamycin in the vesicles both before and after dehydration/rehydration, as well as the rate of drug leakage from the vesicles, were measured by first passing 100 μ l aliquots of the vesicle suspension over columns (1 ml) of Sephadex G-50 to remove any untrapped material (see WO 86/00238 for further details). Trapped adriamycin was then quantitated by mixing an aliquot of the vesicle suspension with 0.5% Triton X-100 (which disrupted the vesicles and released the trapped drug) and monitoring the absorbance at 480 nm employing a Pye Unicam SP8-200 spectrophotometer. Since the columns tend to trap a small percentage of the liposomes applied thereto, the measured values for the amounts of encapsulated material retained after the dehydration/rehydration process are somewhat lower than the levels actually achieved.

The results of these experiments are shown in Table 1. As shown therein, more than 90% of the drug is retained following dehydration and rehydration, i.e. the same levels as those achieved with $^{22}\text{Na}^+$ and ^3H -inulin. Moreover, the rate of leakage of adriamycin from the rehydrated vesicles is comparable to the rate observed with vesicles which have not been dehydrated (see Bally, et al., (1985), *Biochim. Biophys. Acta.*, 812:66).

As clearly demonstrated by this Example, the sugar trehalose is capable of protecting liposomes during dehydration and subsequent rehydration so that more than 90% of the material encapsulated within the liposomes is still retained therein after rehydration.

Example 2

Exposure of Both the Inside and Outside Surfaces of Liposome Membranes to a Protective Sugar

This example illustrates the enhanced protective effect achieved by having a protective sugar (trehalose) in contact with both the internal and external surfaces of the liposome membranes.

ETVs were prepared with trehalose on both sides of the membrane (by including trehalose in the solute solution used to form the vesicles) or only on the outside of the membrane (by excluding trehalose from the solute solution and adding it to the external medium after the vesicles had been formed). The vesicles were dehydrated and at various points in time, up to 72 hours, samples were rehydrated and the level of $^{22}\text{Na}^+$ retained determined. The results are shown in Table 2, together with values for the amount of residual water present in the samples following dehydration.

As shown by this table, maximum protection is achieved when the protective sugar is present on both membrane surfaces. Also, when only the external surface is exposed to the protective sugar, the amount of structural damage which the vesicles undergo is related to the amount of residual water present in samples.

Example 3

Effects of Vesicle Size And Salt Concentration

This example describes the effects of various vesicle sizes and salt concentrations on the ability of trehalose to protect vesicles during dehydration and rehydration.

ETVs of various sizes were produced using polycarbonate filters having pore sizes ranging from 50 nm to 800 nm. The ETVs were subjected to a freeze-thaw cycle as described above in "Materials and Methods." The mean diameters of the resulting vesicles as measured by quasi-elastic light scattering are given in Table 3.

As shown by the data in Table 3, the ability to retain $^{22}\text{Na}^+$ is relatively insensitive to vesicle size. Moreover, since the larger vesicles contained some multilamellar structure, this data illustrates that sugar protection is achieved for multilamellar vesicles. Although the data in Table 3 indicates that the most stable vesicles would appear to be those with a mean diameter of about 170 nm, the multilamellar structure of the

larger vesicles makes a rigorous comparison difficult.

The effects of varying the salt concentration of the internal and external media for a fixed trehalose concentration is shown in Figure 5. As shown therein, there is a small but significant increase in the amount of $^{22}\text{Na}^+$ retained in the vesicles with higher salt concentrations.

Example 4

Relative Ability of Trehalose and Other Sugars to Protect Vesicles during Dehydration

This example illustrates the relative ability of trehalose and other sugars to protect vesicles during dehydration.

Vesicles were prepared in 500 mM trehalose, maltose, lactose, sucrose, and glucose and the amount of $^{22}\text{Na}^+$ retained by the vesicle following dehydration and rehydration determined. As shown in Table 4, trehalose and sucrose were the most effective followed by maltose, glucose and lactose.

Example 5

Dehydration of Liposomes Using the Protective Sugar Streptomycin

This example illustrates the ability of the sugar streptomycin to protect liposomes from substantial loss of their internal contents during dehydration and subsequent rehydration. Experiments demonstrating high retention of ^3H -inulin were performed.

In particular, egg phosphatidylcholine monophasic vesicles (MPVs) were prepared as described in copending and commonly assigned US-A-4 588 578, filed August 8, 1983, and entitled "Lipid Vesicles Prepared in a Monophase." A complete description of the technique used in this example appears in that application.

Briefly, 127 umoles of egg phosphatidylcholine were used in the vesicle preparation. ^3H -inulin and various concentrations of streptomycin were added to phosphate buffered saline (PBS) lacking divalent cations (pH 7.3), and MPVs were formed. The MPVs were dehydrated with prior freezing, rehydrated in PBS and assayed for retained ^3H -inulin as described above. The results are shown in Table 5.

As shown in this table, the amount of ^3H -inulin retained in the vesicles after rehydration depends on the streptomycin concentration, with up to 86% of the inulin being retained at the highest concentration of streptomycin tested (568 mM).

The ability of dihydrostreptomycin to protect liposomes during dehydration/rehydration was also tested following essentially the same protocol as that used with streptomycin except that washing of the MPVs was done with PBS lacking dihydrostreptomycin so that dihydrostreptomycin was only included as part of the internal contents of the finished liposomes. In this case a retention level of 63% was observed for a dihydrostreptomycin concentration level of 565 mM.

Example 6

Loading of Rehydrated Liposomes Using Transmembrane Potentials

This example illustrates: 1) that liposomes having a concentration gradient across their membranes can be dehydrated in the presence of a protective sugar and rehydrated without loss of the concentration gradient; and 2) that after rehydration, the concentration gradient can be used to load a charged material (the drug adriamycin) into the liposomes.

Vesicles having a Na^+/K^+ chemical gradient across their membranes were prepared by forming ETVs in a potassium glutamate buffer (169 mM potassium glutamate, 250 mM trehalose, 20 mM HEPES, pH 7.4), and then replacing the external buffer with a NaCl buffer (150 mM NaCl, 250 mM trehalose, 20 mM HEPES, pH 7.4) by passing the vesicles through a Sephadex G-50 (fine) column (1.4 x 10 cm) which had been pre-equilibrated with the NaCl solution. Where employed, valinomycin (Sigma, St. Louis, Missouri) was added in ethanol to a concentration of 0.5 ug/umole phospholipid.

Similarly, transmembrane pH gradients (interior acid) were formed by preparing the liposomes in a buffer with low pH (135 mM glutamic acid, 250 mM trehalose, brought to pH 5.5 by the addition of potassium hydroxide) which was then exchanged with a high pH buffer (125 mM glutamic acid, 30 mM NaCl, 250 mM trehalose, brought to pH 7.5 by the addition of potassium hydroxide) on a Sephadex G-50 (fine) column. Where used, the proton ionophore CCCP was added to a final concentration of 20 uM.

Transmembrane potentials were measured by determining the distribution of the lipophilic cation ^3H -tetraphenylphosphonium bromide (^3H -TPPB, NEN, Canada). Specifically, 1 μCi of ^3H -TPPB in 1 μl ethanol was added to a 1-2 ml sample of the ETV dispersion and the mixture was incubated at 20°C for 20 minutes. An aliquot (100 μl) was withdrawn and the untrapped ^3H -TPP $^+$ was removed by loading the aliquot onto a Sephadex G-50 column packed in a 1 ml disposable syringe, and then centrifuging the column at 500 g for 3 minutes to elute the vesicles. The trapped ^3H -TPP $^+$ was determined by liquid scintillation counting, and the phospholipid determined by phosphate assay.

Using trapped volume values (μl per μmol of phospholipid) for the ETVs determined by measuring the amount of ^{22}Na or ^3H -inulin captured in the ETVs by the ETV process, the concentrations of ^3H -TPP $^+$ inside $[\text{}^3\text{H}\text{-TPP}^+]_i$ and outside $[\text{}^3\text{H}\text{-TPP}^+]_o$ the vesicles were calculated, from which the transmembrane potential (V_m) was calculated using the Nernst equation:

$$V_m = -59 \log [\text{}^3\text{H}\text{-TPP}^+]_i / [\text{}^3\text{H}\text{-TPP}^+]_o.$$

Both the Na^+/K^+ and the pH gradient vesicles were dehydrated under high vacuum for 24 hours and then rehydrated. Control vesicles were kept at 4°C for 24 hours. Following drying and rehydration, the transmembrane potentials exhibited by these vesicles in the presence and absence of ionophores were compared to the transmembrane potentials generated by the controls, also in the presence and absence of ionophores. The results are shown in Figures 6 (pH) and 7 (Na^+/K^+).

As can be seen from these figures, the transmembrane potentials exhibited by the vesicles which had been dehydrated and then rehydrated are essentially identical to those exhibited by the controls. The only apparent difference is that in the case of the pH gradient vesicles, the transmembrane potentials for the dehydrated/rehydrated vesicles develop somewhat slower than the transmembrane potentials for the control vesicles.

The ability of the Na^+/K^+ vesicles to accumulate adriamycin after dehydration and rehydration was tested in the presence and absence of the ionophore valinomycin, and compared with the accumulation exhibited by the control vesicles, i.e., the vesicles which had been stored at 4°C for 24 hours rather than being dehydrated for 24 hours. Sufficient adriamycin was added to the vesicles' external medium to produce a final concentration of 0.2 moles adriamycin per mole of phospholipid.

The results of these tests are shown in Figure 8. As can be seen therein, the dehydrated/rehydrated vesicles accumulate adriamycin essentially at the same rate and to the same extent as the control vesicles.

Although not wishing to be bound by any particular theory of operation, one of the mechanisms involved in the observed uptake of adriamycin in response to Na^+/K^+ gradients may involve the pH gradients which are automatically generated in response to such gradients due to the permeability of liposome membranes to H^+ ions. In accordance with this mechanism, adriamycin passes through the membrane in an uncharged state, with its internal and external concentrations being a function of the internal and external H^+ ion concentrations, the internal concentration being high when the internal H^+ concentration is high, and vice versa.

In sum, this example demonstrates that delayed loading of vesicles can be accomplished through the combination of concentration gradients and the dehydration/rehydration process.

TABLE 1

Ability of Dehydrated Vesicles to Retain Adriamycin	
	Adriamycin Content (nmoles/ μmole lipid)
Before dehydration	197
Immediately after dehydration and rehydration	185
One hour after dehydration and rehydration	158
Two hours after dehydration and rehydration	145
ETVs were prepared using a solute solution containing adriamycin and 250 mM trehalose. The samples were dehydrated for 24 hours without prior freezing. The adriamycin content of the initial sample and the rehydrated vesicles was determined as described in Example 1.	

TABLE 2

Sample	Drying Time (Hrs.)	$^{22}\text{Na}^+$ retained %	Residual water %
Trehalose on both sides of membrane (250 mM)	24	94	5.6
	48	84	5.4
	72	84	5.2
Trehalose on outside of membrane (250 mM)	24	68	5.4
	48	49	5.0
	72	17	4.2

TABLE 3

Influence of Vesicle Size on Ability to Retain $^{22}\text{Na}^+$		
Polycarbonate filter pore size (nm)	Vesicle mean diameter (nm)	% $^{22}\text{Na}^+$ retained following dehydration and rehydration
800	500	80%
400	220, 500	84%
200	180, 375	87%
100	170	92%
50	112	88%
Vesicles were prepared with varying mean diameters by extruding multilamellar vesicles through polycarbonate filters of appropriate diameters (see "Materials and Methods"). The samples all contained 250 mM trehalose and were dehydrated for 24 hours without prior freezing.		

TABLE 4

Comparison of the Ability of Different Sugars To Stabilize Vesicles in the Anhydrous State	
Sugar tested (500 mM)	% $^{22}\text{Na}^+$ retained following dehydration and rehydration
Trehalose	88%
Glucose	73%
Sucrose	86%
Maltose	76%
Lactose	66%
Large unilamellar vesicles were prepared in the presence of 500 mM of each sugar, dehydrated for 24 hours without prior freezing, and the amount of trapped $^{22}\text{Na}^+$ retained upon rehydration determined as described in "Materials and Methods."	

TABLE 5

Ability of Streptomycin to Protect Vesicles During Dehydration-Rehydration		
	% ³ H-Inulin Retained	
	Control	Freeze-Dried
PBS	97	36
PBS plus 52 mM streptomycin	92	31
PBS plus 568 mM streptomycin	94	86
Monophasic vesicles were prepared and washed in the presence and absence of streptomycin, dehydrated under vacuum with prior freezing, and rehydrated as described in Example 5. The control vesicles were stored at room temperature for the same periods of time as used for the freeze-drying process (24 hours).		

TABLE 6

Inulin Retention as a Function of
Vesicle Type, Lipid Type and Lipid Concentration

<u>Lipid Concentration</u>	<u>Vacuum Dessication</u>	<u>Lyophilization</u>	<u>Control</u>
<u>MPV</u>			
EPC (low)	47.0 ± 0.5	27.1 ± 3.1	86.6 ± 6.1
EPC (high)	63.9 ± 6.0	58.3 ± 1.7	101.3 ± 1.8
SPC (low)	41.0 ± 3.5	29.2 ± 5.3	71.7 ± 10.1
SPC (high)	54.6 ± 5.4	42.4 ± 1.8	96.9 ± 2.2
<u>MLV</u>			
EPC (low)	44.6 ± 14.7	40.7 ± 24.6	N.D.
EPC (high)	62.0 ± 3.8	55.4 ± 5.8	99.8 ± 2.1
SPC (low)	28.4 ± 10.1	18.3 ± 3.9	N.D.
SPC (high)	60.4 ± 2.2	53.8 ± 3.3	98.4 ± 7.8
<u>SPLV</u>			
EPC (low)	31.3 ± 0.6	17.0 ± 3.8	69.3 ± 7.5
EPC (high)	56.4 ± 1.9	54.4 ± 3.5	98.5 ± 2.8
SPC (low)	37.2 ± 2.9	24.6 ± 0.2	87.6 ± 1.0
SPC (high)	48.4 ± 6.2	44.6 ± 2.3	97.8 ± 1.7
<u>FATMLV</u>			
EPC (low)	35.6 ± 4.2	18.4 ± 2.6	102.1 ± 4.9
EPC (high)	54.9 ± 0.1	34.8 ± 7.7	95.3 ± 0.4
SPC (low)	24.6 ± 12.2	24.4 ± 1.6	89.8 ± 11.4
SPC (high)	43.7 ± 6.0	27.9 ± 0.7	96.1 ± 1.4

Values reported = % of original inulin retained in the preparation after dehydration and rehydration ± S.D.

N.D. = Not determined.

Low lipid concentration = 3 umol lipid hydrated with 1 ml prior to dehydration.

High lipid concentration = 80 umol lipid hydrated with 1 ml prior to dehydration.

Drying Time - Vacuum Dessication -- 2 days

- Lyophilization -- 1 day

(residual water levels after dehydration equaled approximately 2% or less for both procedures)

Number of experiments per data point = 2; data corrected for blank, 2 channel spillover; EPC = Egg PC; SPC = Soy PC.

Claims

1. A method for dehydrating liposomes comprising the steps of
 - (a) preparing a liposome which includes one or more protective sugars and



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(residual water levels after dehydration equaled approximately 2% or less for both procedures)

Number of experiments per data point = 2; data corrected for blank, 2 channel spillover; EPC = Egg PC; SPC = Soy PC.

Claims

1. A method for dehydrating liposomes comprising the steps of
 - (a) preparing a liposome which includes one or more protective sugars and



(b) removing water from the preparation, wherein said sugars are present at protective concentrations at both the inside and outside surfaces of the liposome membranes, and wherein the liposome substantially retains its contents, or retains its permeability barrier throughout the dehydration and rehydration process, and wherein the liposomes are large unilamellar vesicles.

2. The method for dehydrating liposomes as claimed in claim 1 wherein the liposomes have a concentration gradient of one or more charged species across their membranes, said concentration gradient being capable of generating a transmembrane potential which will load an ionizable bioactive agent into the liposomes.
3. A method according to claims 1 or 2 wherein the concentration of sugar is at least 5 moles of sugar per mole of phospholipid.
4. The method of claims 1 to 3 wherein the one or more protective sugars are disaccharide sugars.
5. The method of claims 1 to 3 wherein the one or more protective sugars are selected from the group consisting of trehalose, maltose, sucrose, glucose, lactose and dextran.
6. The method of any of claims 1 to 3 wherein the one or more protective sugars are aminoglycosides.
7. The method of claim 6 wherein the sugar is streptomycin or dihydrostreptomycin.
8. Liposome preparation which is a dehydrated liposome prepared in accordance with the method of any of claims 1 to 7.
9. The liposome preparation of claim 8 wherein the internal contents comprise a drug.
10. The liposome preparation of claim 8 wherein the drug comprises an antineoplastic agent.
11. The liposome preparation of claim 8 wherein the antineoplastic agent comprises doxorubicin.
12. The liposome preparation of claim 8 wherein the internal content doesn't comprise a drug.

Patentansprüche

1. Verfahren zur Dehydratisierung von Liposomen, umfassend die Stufen
 - (a) Herstellung eines Liposoms, das einen oder mehrere Schutzzucker enthält, und
 - (b) Entfernung von Wasser aus der Zubereitung, wobei die Zucker mit Schutzkonzentrationen sowohl an der inneren als auch an der äußeren Oberfläche der Liposommembranen vorhanden sind, und worin das Liposom im wesentlichen seinen Inhalt beibehält oder seine Durchlässigkeitssperre über den Dehydratisierungs- und Rehydratisierungsprozeß beibehält, und wobei die Liposomen große unilamellare Vesikel sind.
2. Verfahren zur Dehydratisierung von Liposomen nach Anspruch 1, worin die Liposomen einen Konzentrationsgradienten von einem oder mehreren geladenen Spezies über ihre Membranen aufweisen, wobei der Konzentrationsgradient zur Erzeugung eines Transmembranpotentials in der Lage ist, das ein ionisierbares bioaktives Mittel in die Liposomen einträgt.
3. Verfahren nach Anspruch 1 oder 2, worin die Konzentration des Zuckers wenigstens 5 Mole Zucker pro Mol Phospholipid beträgt.
4. Verfahren nach Anspruch 1 bis 3, worin der eine Schutzzucker ein Disaccharidzucker ist oder mehrere Schutzzucker Disaccharidzucker sind.
5. Verfahren nach Anspruch 1 bis 3, worin der eine oder mehrere Schutzzucker aus der Gruppe ausgewählt ist/sind, die aus Trehalose, Maltose, Saccharose, Glucose, Lactose und Dextran besteht.



6. Verfahren nach einem der Ansprüche 1 bis 3, worin der eine Zucker ein Aminoglycosid ist oder mehrere Zucker Aminoglycoside sind.
7. Verfahren nach Anspruch 6, worin der Zucker Streptomycin oder Dihydrostreptomycin ist.
8. Liposompräparation, die ein dehydratisiertes Liposom ist, hergestellt gemäß dem Verfahren nach einem der Ansprüche 1 bis 7.
9. Liposompräparation nach Anspruch 8, worin der innere Inhalt ein Arzneimittel umfaßt.
10. Liposompräparation nach Anspruch 8, worin das Arzneimittel ein antineoplastisches Mittel umfaßt.
11. Liposompräparation nach Anspruch 8, worin das antineoplastische Mittel Doxorubicin umfaßt.
12. Liposompräparation nach Anspruch 8, worin der innere Inhalt kein Arzneimittel umfaßt.

Revendications

1. Procédé pour la déshydratation de liposomes, comprenant les étapes de (a) préparation d'un liposome qui contient un ou plusieurs sucres protecteurs et (b) élimination de l'eau de la composition, dans lequel lesdits sucres sont présents à des concentrations protectrices à la fois sur les surfaces internes et externes des membranes de liposome, et dans lequel le liposome conserve pratiquement son contenu ou conserve sa barrière de perméabilité pendant tout le processus de déshydratation et de réhydratation, et dans lequel les liposomes sont de grandes vésicules unilamellaires.
2. Procédé pour la déshydratation de liposomes selon la revendication 1, dans lequel les liposomes ont un gradient de concentration d'une ou de plusieurs espèces chargées à travers leurs membranes, ledit gradient de concentration étant capable d'engendrer un potentiel transmembranaire permettant d'introduire un agent bioactif ionisable dans les liposomes.
3. Procédé selon la revendication 1 ou 2, dans lequel la concentration de sucre est d'au moins 5 moles de sucre par mole de phospholipide.
4. Procédé selon les revendications 1 à 3, dans lequel le sucre ou les sucres protecteurs sont des disaccharides.
5. Procédé selon les revendications 1 à 3, dans lequel le sucre ou les sucres protecteurs sont choisis parmi le tréhalose, le maltose, le saccharose, le glucose, le lactose et le dextrane.
6. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le sucre ou les sucres protecteurs sont des aminoglycosides.
7. Procédé selon la revendication 6, dans lequel le sucre est la streptomycine ou la dihydrostreptomycine.
8. Composition de liposome qui est un liposome déshydraté préparé conformément au procédé selon l'une quelconque des revendications 1 à 7.
9. Composition de liposome selon la revendication 8, dans laquelle le contenu interne comprend un médicament.
10. Composition de liposome selon la revendication 8, dans laquelle le médicament comprend un agent antinéoplasique.
11. Composition de liposome selon la revendication 8, dans laquelle l'agent antinéoplasique comprend la doxorubicine.
12. Composition de liposome selon la revendication 8, dans laquelle le contenu interne ne comprend pas de médicament.