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(54) Title: A BILAYER STRUCTURE WHICH ENCAPSULATES MULTIPLE CONTAINMENT UNITS AND USES THEREOF

(57) Abstract

The present invention provides a bilayer structure for encapsulating multiple containment units. These containment units can attach or contain therapeutic or diagnostic agents which can be released through the bilayer structure. A suitable example of such a containment unit is a unilamellar or multilamellar vesicle.

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A BILAYER STRUCTURE WHICH ENCAPSULATES MULTIPLE CONTAINMENT UNITS AND USES THEREOF

BACKGROUND OF THE INVENTION

Conventional drug delivery technology, which in the past has concentrated on improvements in mechanical devices such as implants or pumps to achieve more sustained release of drugs, is now advancing on a microscopic and even molecular level. Recombinant technology has produced a variety of new potential therapeutics in the form of peptides and proteins and these successes have spurred the search for newer and more appropriate delivery and targeting methods and vehicles.

Microencapsulation of drugs within biodegradable polymers and liposomes has achieved successes in improving the pharmacodynamics of a variety of drugs such as antibiotics and chemotherapeutic agents. For example, unilamellar vesicles are currently used as drug delivery vehicles for a number of compounds where slow, sustained release, or targeted release to specific sites in the body are desired. The drug to be released is contained within the aqueous interior of the vesicle and release is achieved by slow permeation through the vesicle bilayer. A variety of modifications of the unilamellar vesicle membrane have been attempted, including polymerizing or crosslinking the molecules in the bilayer to enhance stability and reduce permeation rates, and incorporating polymers into the bilayer to reduce clearance by macrophages in the bloodstream.

One example of such a vesicle structure is known as Depofoam. Depofoam is a multivesicular particle which is created by multiple emulsification steps. A defined lipid composition is dissolved in a volatile solvent. The dispersed lipids in solvent are vigorously mixed with water to form a first emulsion, designated a solvent continuous emulsion.

This first emulsion is then added to a second water/solvent emulsion and emulsified to form a water in solvent in water double emulsion. The solvent is removed from the mixture resulting in discrete foam-like spherical structures consisting of bilayer separated water compartments. The minimum size of these structures is about 5-10 microns. Depofoam does not include a distinct bilayer structure which encapsulates the multivesicular particles, i.e., there are no individual, distinct interior vesicles. Therefore, the interior compartment must share bilayer walls.

Liposomes are sealed, usually spherical, either unilamellar or multilamellar vesicles which are capable of encapsulating a variety of drugs. Liposomes are the most widely studied vesicles to date and they can be formulated with a variety of lipid types and compositions which can alter their stability, pharmacokinetics and biodistribution. A major disadvantage of both multilamellar and unilamellar liposomes as delivery systems is their size, which prevents them from crossing most normal membrane barriers and limits their administration to the intravenous route. In addition, their tissue selectivity is limited to the reticuloendothelial cells, which recognize them as foreign microparticulates and then concentrates the liposomes in tissues such as the liver and spleen.

Polymers have also been used as drug delivery systems. They generally release drugs by (1) polymeric degradation or chemical cleavage of the drug from the polymer, (2) swelling of the polymer to release drugs trapped within the polymeric chains, (3) osmotic pressure effects, which create pores that release a drug which is dispersed within a polymeric network, and/or (4) simple diffusion of the drug from within the polymeric matrix to the surrounding medium.

With the success and drawbacks of these microencapsulation vehicles, today the challenge is to produce better and more efficient microencapsulation vehicles to enhance drug delivery. The present invention is directed to meeting that challenge.

SUMMARY OF THE INVENTION

The present invention provides a bilayer structure for encapsulating multiple containment units, e.g., polymer containment units. Containment units can attach or contain therapeutic or diagnostic agents which can be released through the bilayer structure. A suitable example of such a containment unit is a unilamellar or multilamellar vesicle.

The invention provides compositions comprising the bilayer structure of the invention and multiple containment units. Preferably, the containment units are aggregated.

In one embodiment of the invention, these compositions include vesosomes. Vesosomes have a bilayer structure which encapsulates multiple containment units in the form of vesicles. Generally, the multiple vesicles are aggregated. Vesicles can be unilamellar or multilamellar.

The vesicles can either be of similar size and composition or of varied size and composition. Preferably, each vesicle is attached to another (or aggregated) via ligand-receptor or antibody-antigen interactions.

Further, the encapsulating bilayer structure can attach to the aggregated vesicles via ligand-receptor interactions. By optimizing both the exterior bilayer structure and the interior vesicle compositions, the size and size distribution of the interior vesicles, the overall size of the vesosome, the nature of the attachments of the vesicles, and the type of additives to the outer bilayer (such as polymers or specific recognition sites), an extremely versatile drug delivery system can be developed for a variety of applications.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a freeze-fracture transmission electron micrograph of a typical vesosome prepared upon mixing cochleated cylinders with sized vesicle aggregates (at a 1:1 mole ratio) prior to the addition of EDTA. There is only

one outer bilayer, and the interior vesicles appear to be specifically aggregated.

Figure 2 is a freeze-fracture transmission electron micrograph of a typical vesosome prepared upon mixing cochleated cylinders with active sized aggregates (at a 1:1 mole ratio) after addition of EDTA. Again, there is only one outer bilayer, and the interior vesicles appear to be specifically aggregated. The two figures differ only in that EDTA has been added to the second sample to chelate the remaining calcium.

Figure 3 is a schematic representation of one embodiment for vesosome production.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS:

As used in this application, the following words or phrases have the meanings specified.

As used herein, the term "containment units" means any structure having space which can be occupied by an agent such as a therapeutic, diagnostic, or cosmetic agent. Generally, the structure is spherical but is not necessarily so.

As used herein the term "vesosome" is an aggregate of unilamellar or multilamellar vesicles encapsulated within a distinct bilayer structure. The interior vesicles can be of a single size and membrane or interior composition or number of layers, or of varied sizes and/or membrane or interior composition or number of layers. These parameters can be controlled during the assembly process described herein.

As used herein "uniform size" means of approximately similar size. It does not necessarily mean vesicles having an identical size.

In order that the invention herein described may be more fully understood, the following description is set forth.

COMPOSITIONS OF THE INVENTION

The invention provides a bilayer structure for encapsulating multiple containment units.

The present invention further provides a vesosome having the bilayer structure of the invention which encapsulates multiple vesicles. Generally, the vesicles are aggregated within the bilayer structure (S. Chiruvolu et al. (1994) Science **264**:1753). The interior vesicles can be of a single size and membrane or interior composition, or of varied sizes and/or membrane or interior composition. The interior vesicles can either be unilamellar or multilamellar. These parameters can be determined during the assembly process described below. The total dimensions of the vesosome can be controlled from about 0.05 micron to >5 micron. The vesosome can incorporate a variety of water or lipid soluble drugs or other solutes within the interior vesicles, or within the exterior capsule, or both. These drugs can then permeate slowly through the interior and exterior bilayers, providing a controlled, slow release of drugs over time. Aggregation can be effected by ligand-receptor or antibody-antigen interactions. Other aggregation means are possible.

The vesosome of the invention is submicroscopic in size. Further, in accordance with the practice of the invention, the multiple vesicles are of uniform size. Alternatively, the multiple vesicles are of dramatically different sizes.

METHODS FOR USING COMPOSITIONS OF THE INVENTION

The invention further provides a method for encapsulating multiple vesicles within an outer membrane. This method comprises obtaining aggregated multiple vesicles in a solution. Cochleated cylinders are then added to the

solution [Papahadjopoulos, D., et al., (1974) *Biochim. Biophys. Acta,* **401**, 317-335; Papahadjopoulos, D., et al., (1975) *Biochim. Biophys. Acta,* **394**, 483-491; Papahadjopoulos, D., et al., (1976) *Biochim. Biophys. Acta,* **448**, 265-283].

The aggregated vesicles and cochleated cylinders are mixed in the solution under suitable conditions so that the cochleated cylinders transform to create the bilayer structures of the invention which encapsulates the aggregated multiple vesicles.

In accordance with the practice of the invention, the interior vesicles can include a therapeutic agent. Alternatively, the interior vesicles can include a diagnostic agent. The interior vesicles can include a reactive agent, so as to create new compounds in situ. Methods for including agents within the containment units (e.g., vesicles) are well known because methods for including such agents into liposomes are well known (CA 1314209; DE 3880691; GB 9605915; DE 4402867).

Suitable therapeutic agents include, but are not limited to, the following.

The therapeutic agent can include antimicrobial agents such as antibiotics, antifungal, and antimycobacterial drugs. Examples of antibiotics include, but are not limited to, amikacin, kanamycin B, amphomycin, bacitracin, bicyclomycin, capreomycin, polymyxin E, cycloserine, chloramphenicol, dactinomycin, erythromycin, gentamicin, gramicidin A, penicillins, rifamycins, streptomycin, and tetracyclines.

The therapeutic agent can be a drug acting at synaptic and neuroeffector junctional sites. Examples include neurohumoral transmitters, cholinergic agonists, anticholinesterase agents, antimuscarinic drugs, agents acting at the neuromuscular junction and autonomic ganglia, catecholamines, sympathomimetic drugs, and adrenergic receptor antagonists.

Alternatively, the therapeutic agent can be a drug acting on the CNS. Examples include antipsychotic drugs, neuroleptic drugs, tricyclic

antidepressants, monoamine oxidase inhibitors, lithium salts, and benzodiazepines.

Additionally, the therapeutic agent can be a drug which reduces inflammation. Exmples include antagonists of histamine, bradykinin, 5-hydroxytryptamine; lipid-derived autacoids; methylxanthines, cromolyn sodium; and analgesic-antipyretics.

The therapeutic agent can be a drug which affects renal function and electrolyte metabolism. Examples include diuretics and inhibitors of tubular transport of organic compounds.

The therapeutic agent can be a drug which affects cardiovascular function. Examples include renin and angiotensin; organic nitrates, calcium-channel blockers and beta-adrenergic antagonists; antihypertensive agents, digitalis, antiarrhythmic drugs, and drugs used in the treatment of hyperlipoproteinemias.

Suitable diagnostic agents include, but are not limited to, radiolabels, enzymes, chromophores and fluorescers.

In accordance with the practice of the invention, the aggregated multiple vesicles of step (a) of the method and the cochleated cylinders of step (b) of the method can be mixed in a suitable ratio, e.g., a 1:1 ratio. Other ratios are possible.

The present invention provides a method for delivering a therapeutic agent to a wound site. This method comprises introducing the vesosome of the invention to the wound site. This is done under conditions so that the therapeutic agent is released from the vesosome to the wound site. Alternatively, the method also include delivering an agent to an intended site for cosmetic, vetinary, and other applications requiring slow release of a particular agent (CA 1314209; DE 3880691; GB 9605915; DE 4402867).

Introduction of the vesosome to the wound site can be effected by various methods. For example, the vesosome can be introduced by intramuscular injection, intravenous injection, oral administration, pulmonary adsorption, rectal administration, subcutaneous injection, sublingual administration, or topical application. Other methods are administration methods are possible and well known in the art.

The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the severity and course of the disease, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

The interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Ferrite, E.J., et al. (Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey and Man. Cancer Chemother, Rep., 50, No.4, 219-244, May 1966).

Adjustments in the dosage regimen can be made to optimize the response. Doses can be divided and administered on a daily basis or the dose can be reduced proportionally depending upon the situation. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the specific therapeutic situation.

The invention further provides a method for obtaining aggregated vesicles having a uniform size. As used herein, "uniform size" means having about the same, but not necessarily having identical, size.

This method comprises filtering aggregated vesicles having varying sizes through multiple membranes under pressure. By doing so, the vesicle aggregates so filtered have substantially uniform size. For example, the vesicles so extruded can have a size ranging from 0.05-5 μm.

The vesicles are filtered through two membranes, although this is not essential to the process. Generally, the membranes have filter pores of uniform size.

Possible Modifications and Variations

As mentioned, the detailed composition and size of the interior vesicles is not important to the process. Other types of vesicle preparation methods can be used, from (1) chemical preparations such as reverse phase evaporation, detergent dialysis, pH jump, (2) other mechanical treatments such as ultrasonication, and (3) spontaneous vesicle preparations which lead directly to equilibrium vesicles (without special treatments). Unencapsulated drug product can be removed at any stage of this process by various dialysis techniques, ion exchange, chromatography, filtration or centrifugation.

The aggregation of the vesicles can also be accomplished by a variety of ligand -receptor interactions, via antigen-antibody, or via chemical crosslinking agents that mimic ligand-receptor interactions.

Aggregate sizing can be accompished by other methods such as (1) quenching the aggregation (adding another ligand that binds to the receptor, preventing it from cross-linking more vesicles), (2) using charged vesicles that will aggregate at a slower rate due to enhanced electrostatic repulsion between the vesicles and (3) altering the stoichiometric ratio of the ligand to receptor, which also can lead to a slower, controlled aggregation. Monodisperse aggregate sizes can be prepared by removing excess freely floating vesicles or small aggregates by various centrifugation or dialysis techniques. Finally, polymers such as polyethylene glycol linked to lipids can be incorporated into the exterior membrane to sterically stabilize the vesosome against aggregation and/or clearance by macrophages. The various processes can be optimized for a particular drug release or other application.

There are also different methods of forming the exterior membrane in addition to the method described above. The simplest and most general is to

use a layer of large vesicles attached to the aggregated vesicles, then osmotically stress these exterior vesicles to "pop" them, thereby forming a continuous encapsulating membrane in the process. A second method is to use large, flaccid vesicles to engulf the aggregated vesicles in a type of endocytosis process, again mediated by ligand receptor or similar interactions.

Advantages of the invention: The benefits of the vesosome over single walled vesicles used for drug delivery is that important membrane functions can be divided among two or more membranes rather than one. For example, the permeation rate, the membrane charge, specific recognition molecules, steric stabilizers, membrane rigidity and phase transition temperatures all play a role in the optimization of a drug delivery vehicle.

With the vesosome structure, these often incompatible attributes can be divided among the various membranes. The exterior membrane can incorporate steric stabilizer molecules such as polyethylene glycol, or specific recognition sites such as ligand or specific receptors. The interior vesicles can be made of various sizes and compositions to optimize the permeation of drugs from the vesosome. The interior vesicles within the vesosome can be of different composition or size from each other as well to optimize delivery of multiple drugs, or prolong delivery over time.

Building the vesosome takes advantage of several new features developed in our laboratory including:

1) Specific aggregation of vesicles via ligand-receptor interactions (Chiruvolu et al., 1994)

2) Sizing the vesicle aggregates via extrusion

3) Encapsulating the vesicles within a second membrane. As far as we have been able to determine from the scientific literature, this is the first time vesicles have been encapsulated within another bilayer by a controlled and reproducible process [Cevc, G. and Marsh, D. (1987) *Phospholipid Bilayers: Physical Principles and Models*, Wiley, New York; Lasic, D. D. (1993) *Liposomes: From Physics to Applications*, Elsevier, Amsterdam].

The greatest benefit to this step-wise process of construction is the great flexibility it allows in optimizing bilayer composition, aggregate size, etc. Also, as many of the steps in the process are spontaneous self-assembly steps, they are especially simple and only involve mixing one or more solutions. As a result, these steps are quite efficient and easy to scale up.

The following example is presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The example is not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

The preparation of the vesosome is essentially a two-step process (Figure 3). The first step is creating a controlled-size vesicle aggregate. The second step is encapsulating the vesicle aggregate within an outer membrane. Here we provide a specific example of the techniques used to create the vesosomes shown in Figs. 1 and 2 above. In each case, the specifics of the lipids and crosslinking agents, the size distributions, etc., used are only representative and can be optimized to suit the application.

Vesicle Preparation:

The vesicles can be made from a variety of phospholipids, cholesterol, fatty acids, etc. as needed. To create the vesosomes shown in Figs. 1 and 2, 150 mg of dilauroylphosphatidylcholine (DLPC) (Avanti Polar Lipids, Alabaster, AL) and 0.4 mg of biotin-X-dipalmitoylphosphatidylethanolamine (B-DPPE) (Molecular Probes, Eugene, OR) were mixed together in a 2 dram sample vial in chloroform (the B-DPPE was present at 0.163 mole % of total lipid in solution) to thoroughly mix the lipids. The chloroform was removed by evaporation under vacuum. 5 mL of aqueous buffer/salt/azide solution (100 mM NaCl, 50 mM TES, and 0.02 wt% NaN_3 balanced to pH 7.2) was added to the dried lipid to create a solution of 30 mg/mL total lipid. The sodium azide is used as a preservative, and is not necessary for the process.

After fully hydrating the lipids, the resultant solution consisted of multilamellar vesicles (MLVs). Unilamellar vesicles were formed from the MLV's by a mechanical extrusion technique [Mayer, E. (1985) *J. Microsc.* **140**, 3-15]. The MLV solution was repeatedly (1) frozen in a liquid nitrogen (T = -190¡C) bath for 30-60 seconds, then (2) immediately melted in a 50-60¡C water bath. This process disrupts the multilamellar structure of the vesicles and leads to the formation of large unilamellar vesicles (LUVs; polydisperse, up to a few microns in size). The solution is then allowed to cool to room temperature (25¡C). The LUV suspension is then put through 8-12 high pressure (approximately 50 psi dry nitrogen) extrusion cycles by filtering the solution within an Extruder (Lipex Biomembranes, Vancouver, BC, Canada) through two stacked Nuclepore filters of pore diameter 0.1 μm. This process produces a 30 mg/mL monodisperse population of unilamellar vesicles (ULVs) approximately 100 nm in diameter. These vesicles consist of DLPC and B-DPPE, with B-DPPE being present in the bilayer at 0.163 mole %. The biotin ligand is oriented away from the bilayer (in the same direction as the headgroups). This creates a vesicle which has several ligands protruding from the both the interior and exterior surface. This solution of vesicles is then allowed to equilibrate for at least a few hours. Although metastable, these ULVs remain freely suspended for several weeks without reverting to their equilibrium MLV structure.

Vesicle Aggregate Preparation:

To aggregate the vesicles, an aqueous dispersion of streptavidin molecules (mol. wt. 60,000 g/mol) in the same buffer solution is added to the extruded vesicles. In this example, 3.94 mg of streptavidin (Molecular Probes, Eugene, OR) is measured and mixed with 6.24 mL of the TES/NaCl/azide buffer solution to create a 0.63 mg/mL streptavidin solution. 1.0 mL of streptavidin solution is added to a vial containing 2.0 mL of the DLPC/B-DPPE ULV suspension. The overall biotin-streptavidin mole ratio for this system is about 15:1, however, the ratio of exposed biotin (biotins on the outer vesicle monolayer) to streptavidin is about 8:1. Since there are four identical binding sites of streptavidin available for binding, the ratio of exposed biotins to

binding sites is 2:1. Within an hour, the 20 mg/mL ULV/streptavidin suspension changes color from clear and bluish to opaque and cloudy, indicating that much larger particles are being formed, i.e., the vesicles are aggregating. This vesicle aggregation scheme does not appear to stress or rupture the individual vesicles. This process was developed in our laboratory [Chiruvolu, S., et al., (1994) *Science* **264**, 1753-1756].

Controlled-Size Vesicle Aggregates Preparation:

Vesicle aggregate sizing is done by extruding the large vesicle aggregates through two stacked Nuclepore filters of pore diameter 1.0 μm; this extrusion is essentially identical to the extrusion step in ULV production, except the pore size is larger. This produces a dispersion of vesicle aggregates with sizes ranging from 0.3-1.0 μm. Once formed, the sized vesicle aggregates are stable for weeks and experience minimal re-aggregation or re-dispersion.

Encapsulating Sized Aggregates

To encapsulate the vesicle aggregate, we take advantage of microstructures common to negatively charged lipids in the presence of calcium ions. Cochleated cylinders are multilamellar lipid tubules formed spontaneously by certain negatively charged phospholipids in the presence of calcium ions. Ca^{2+} is know to induce the adhesion, fusion and collapse of bilayers containing large proportions of the anionic phospholipid phosphatidylserine (PS) [Papahadjopoulos, D., et al., (1974) *Biochim. Biophys. Acta,* **401**, 317-335; Papahadjopoulos, D., et al., (1975) *Biochim. Biophys. Acta,* **394**, 483-491; Papahadjopoulos, D., et al., (1976) *Biochim. Biophys. Acta,* **448**, 265-283]. These dehydrated multilamellar structures have been synthesized in our laboratory using similar techniques reported in the literature as discussed below. We have independently confirmed the presence of cochleated cylinders in our experiments by FF-TEM.

DOPS Unilamellar Vesicle Preparation:

Vesicles, composed of 1,2 dioleoylphosphatidylserine (DOPS ; AVANTI Polar Lipids, Inc., Alabaster, AL) and containing small amounts of B-DPPE (Molecular Probes, Inc., Eugene, OR) were made as precursors to cochleated cylinders through similar methods as described above.

Briefly, 50 mg of lyophilized DOPS (61.7 μmoles) was dissolved in 5 mL of Chloroform with 0.1 ml of B-DPPE solution [9.8×10^{-8} mole B-DPPE] to give

a mole fraction of B-DPPE of 0.0016. The chloroform was evaporated under dry nitrogen and the lipid vacuum dried to remove excess solvent. The dried, mixed lipids were then hydrated (or resuspended) in 5 mL aqueous buffer solution as described above, yielding a solution with DOPS (MW 810 g/mol) concentration of 10 mg/mL (12.3 mM) and a B-DPPE (MW 1019 g/mol) concentration of 0.02 mg/mL (0.02 mM).

After dispersing the lipid by vortexing, we allowed equilibration of the solution at 37 ¡C for 24 hours. The multilamellar vesicle solution was taken through several freeze-thaw cycles prior to sizing by high pressure extrusion through Nuclepore 0.1 μm polycarbonate membranes. The sized vesicles were allowed to equilibrate at 25¡C prior to the addition of Ca^{2+}.

Ca2+ Solution Preparation:

Solutions containing millimolar quantities of free Ca^{2+} were prepared using anhydrous $CaCl_2$ salt (Sigma Chemical Co., St. Louis, MO) and the standard buffer solution. Previous experiments by our laboratory revealed that the concentration of Ca^{2+} in solution required to induce fusion between small unilamellar vesicles of DOPS be greater than 2.0 mM. A 6.0 mM $CaCl_2$ buffer solution was prepared for use in these experiments.

Cochleated Cylinders Preparation:

Equal 1 mL volumes of the DOPS/B-DPPE vesicle solution (10 mg/mL) and the 6 mM $CaCl_2$ buffer solution were measured using two 1000 μL Hamilton Gas-Tight® syringes. The two solutions were simultaneously dispensed into a clean, dry 3-dram vial, where they rapidly mixed to form a solution with a DOPS concentration of 5 mg/mL (6.2 mM), a B-DPPE concentration of 0.01 mg/mL (0.01 mM) and a $CaCl_2$ concentration of 3 mM. Immediately upon mixing, the turbidity of the solution increased. Aggregation, fusion and collapse of the DOPS/B-DPPE vesicles -- and transition into cochleated cylinders-- began immediately.

Streptavidin (Molecular Probes, Inc., Eugene OR) was dissolved in the standard buffer for a solution with a concentration 0.63 mg/mL ($1.0x10^{-8}$ mol/mL). 35 μL of the streptavidin solution was injected into 1 mL of the cochleated cylinder solution to activate the cylinders. The product was gently mixed and allowed to equilibrate for 24 hours.

Vesosome Preparation:

We have now described how to prepare the two precursor solutions (in identical buffers) needed for vesosome production. First, we have a solution of active vesicle aggregates (with some active freely floating vesicles). Second, we have a solution of active cochleated cylinders (with likely some freely floating streptavidin).

We have employed two different mixing ratios of the two precursor solutions that produce the vesosome solutions. To briefly describe, one mixture is prepared such that the ratio of the number of moles of DLPC lipids to DOPS lipids equals one. The second mixture is prepared such that the ratio of the approximate number of sized vesicle aggregates (taking into account the freely floating vesicles) to the approximate number of cochleated cylinders equals one. In the latter case, we are attempting to match at least one aggregate with one cylinder. In the former case, we are ensuring that there are plenty of aggregates to get encapsulated.

In the mole-match case, 1.0 mL of the 5 mg/mL (DOPS) active cochleated cylinders/streptavidin solution is added to the 20 mg/mL (DLPC) 0.190 mL of active sized vesicle aggregates simultaneously. That is, 6.2 μmol of DOPS molecules is mixed with 6.2 μmol of DLPC molecules. Upon mixing, the solution turned from chunky, crystal-like structures consistent with suspensions of cylinder solutions to more opaque and less chunky.

In the number-match case, 1.0 mL of the 5 mg/mL (DOPS) active cochleated cylinders/streptavidin solution is added to the 20 mg/mL (DLPC) 0.040 mL of active sized vesicle aggregates simultaneously. That is, 6.2 μmol of DOPS molecules is mixed with 1.3 μmol of DLPC molecules. Therefore, in the mole-match case, there are about 5 times as many aggregates as in the number-match case. Again, upon mixing, the nature of the solution changed.

Freeze-fracture TEM Results

Aliquots for freeze-fracture sample preparation were taken from both the mole-match and number-match solutions one day after mixing the cylinders and aggregates.

In general, FF-TEM revealed that most of the structures present in either the mole-matched or number-matched solution were LUVs (1-5 μm). Very few cylinders were observed. There did appear to be some unencapsulated sized

aggregates as well a high concentration of free vesicles (100 nm). Actual concentrations or numbers are not available.

There did exist several vesosomes, as shown in **Fig. 1** The interior aggregated vesicles (1) do appear to resemble the aggregated vesicles in both size (~0.5 μm) and aggregation state (dense and compact) prior to mixing the solutions and (2) are approximately 100 nm in size. These features indicate that the vesicles are indeed the DLPC vesicles and not DOPS vesicles which have managed to remain even after Ca^{2+} addition.

There are, however, a few larger vesicles present within, and these may simply be larger DLPC ULVs or ULVs which have been formed by the fusion of several DLPC ULVs. Also, note that the vesosome has a single bilayer encapsulating the entire vesicle aggregate, consistent with the "unrolling" of a cylinder.

A general encapsulation process could occur as follows: after mixing the solutions, an active aggregate approaches an active cylinder and binds to its surface by at least one biotin-streptavidin interaction. The aggregate proceeds to bind in several places until the binding force overcomes the force necessary to keep the cylinder wound. As the cylinder begins to unwind, the interior regions of the cylinder, now exposed to the aqueous solution, continue to bind around the aggregate until the cylinder unravels completely around the aggregate as if forced by the presence of EDTA.

Next, 0.44 mL of 5 mM EDTA solution (in the same buffer) was added to 0.5 mL of the mole-matched cylinder-aggregate mixture. The cloudy, opaque solution immediately turned grayish and more transparent. This cylinder-aggregate mixture consisted of approximately 4.2 mg/mL DOPS (cylinders) and 3.2 mg/mL DLPC (aggregates). The amount of EDTA added was in excess of the amount necessary to completely bind all of the available calcium ions and therefore cause unraveling of the cylinders. Also, 0.5 mL of the 5 mM EDTA solution was added to the number-matched mixture. Again, the solution changed to grayish and transparent. This number-matched mixture

consisted of 4.8 mg/mL DOPS and 0.8 mg/mL DLPC. Again, excess EDTA was added. Aliquots of each of these solutions were also taken after approximately five hours of incubation for freeze-fracture sample preparation.

FF-TEM again revealed that each of these solutions contained very many LUVs (1-5 μm), as is expected in solutions in which EDTA has been added to cylinders. Also, there were several ULVs but no cylinders.

Vesosomes again were present in the number-match solution. **Fig. 2** shows a typical vesosome seen in these solutions. Note, again, that interior vesicles appear to be aggregated as in the precursor solutions. However, there are also some very large vesicles, probably unraveled DOPS vesicles, which have become encapsulated as well. The number of vesosomes relative to the number of LUVs in these solutions does not seem to vary between the pre- and post-EDTA solutions, however, there seem to be more of them in the mole-matched solutions. This may indicate that the more active particles added to solution increases the chances for a vesosome to form.

It should be noted that the vesosome structures were not present in either of the precursor solutions. The solutions of cylinders saturated with streptavidin did not show any unusual characteristics due to the presence of the streptavidin; in fact, the cylinders seemed to become more dispersed, which may have been due to the bound streptavidin acting like a steric stabilizer, keeping the cylinders isolated from each other. The solutions of sized vesicles also did not exhibit any feature resembling a vesosome. No LUVs were even present in these solutions.

What is claimed is:

1. A composition comprising a bilayer structure and multiple containment units, the bilayer structure of claim 1 encapsulating the multiple containment units.

2. The composition of claim 1, wherein the containment units are aggregated within the bilayer structure.

3. The composition of claim 1, wherein the multiple containment units include a therapeutic agent

4. The composition of claim 1, wherein the multiple containment units include a diagnostic agent.

5. A vesosome having a bilayer structure and multiple vesicles, the bilayer structure encapsulating the multiple vesicles.

6. The vesosome of claim 5, wherein the vesicles are aggregated within the bilayer structure.

7. The vesosome of claim 5, wherein the multiple vesicles are of different size.

8. The vesosome of claim 5, wherein the multiple vesicles are of the same size.

9. The vesosome of claim 5, wherein the multiple vesicles include a therapeutic agent

10. The vesosome of claim 5, wherein the multiple vesicles include a diagnostic agent.

11. A method for encapsulating multiple containment units within a bilayer structure comprising:

a. obtaining aggregated multiple containment units in a solution;

b. adding cochleated cylinders to the solution; and

c. mixing the aggregated multiple containment units of step (a) and the cochleated cylinders of step (b) in the solution under suitable conditions so that the cochleated cylinders transform to create the bilayer structure that encapsulates the aggregated multiple containment units.

12. A method for delivering a therapeutic agent to a target site which comprises introducing the vesosome of claim 8 to the target site under conditions so that the therapeutic agent is released therefrom.

13. The method of claim 12, wherein the vesosome is introduced by intramuscular injection, intravenous injection, oral administration, pulmonary adsorption, rectal, nasal, oral, ocular, vaginal, or uretheral administration, subcutaneous injection, sublingual administration, or topical application.

14. A method for delivering a therapeutic agent to a target site which comprises introducing the composition of claim 3 to the target site under conditions so that the therapeutic agent is released therefrom.

15. The method of claim 14, wherein the composition is introduced by intramuscular injection, intravenous injection, oral administration, pulmonary adsorption, rectal, nasal, oral, ocular, vaginal, or uretheral administration, subcutaneous injection, sublingual administration, or topical application.

16. The method of claim 12 or 14, wherein the therapeutic agent is a drug acting at synaptic and neuroeffector junctional sites.

17. The method of claim 16, wherein the drug is selected from a group consisting of a neurohumoral transmitter, a cholinergic agonist, an anticholinesterase agent, an antimuscarinic drug, an agent acting at the neuromuscular junction and autonomic ganglia, a catecholamine, a sympathomimetic drug, and an adrenergic receptor antagonist.

18. The method of claim 12 or 14, wherein the therapeutic agent is a drug acting on the CNS.

19. The method of claim 18, wherein the drug is selected from a group consisting of an antipsychotic drug, a neuroleptic drug, tricyclic antidepressants, monoamine oxidase inhibitors, lithium salts, and benzodiazepines.

20. The method of claim 12 or 14, wherein the therapeutic agent is a drug which reduces inflammation.

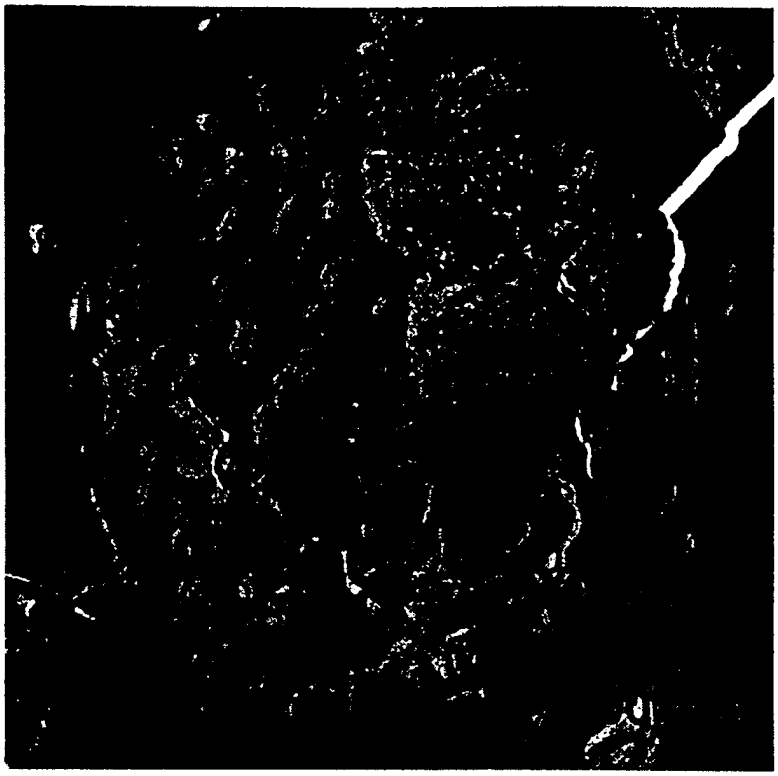
21. The method of claim 20, wherein the drug is selected from a group consisting of antagonists of histamine, bradykinin, 5-hydroxytryptamine; lipid-derived autacoids; methylxanthines, cromolyn sodium; analgesic-antipyretics.

22. The method of claim 12 or 14, wherein the therapeutic agent is a drug which affects renal function and electrolyte metabolism.

23. The method of claim 22, wherein the drug is selected from a group consisting of diuretics and inhibitors of tubular transport of organic compounds.

24. The method of claim 12 or 14, wherein the therapeutic agent is a drug which affects cardiovascular function.

25. The method of claim 24, wherein the drug is selected from a group consisting of renin and angiotensin; organic nitrates, calcium-channel blockers and beta-adrenergic antagonists; antihypertensive agents, digitalis, antiarrhythmic drugs, and drugs used in the treatment of hyperlipoproteinemias.



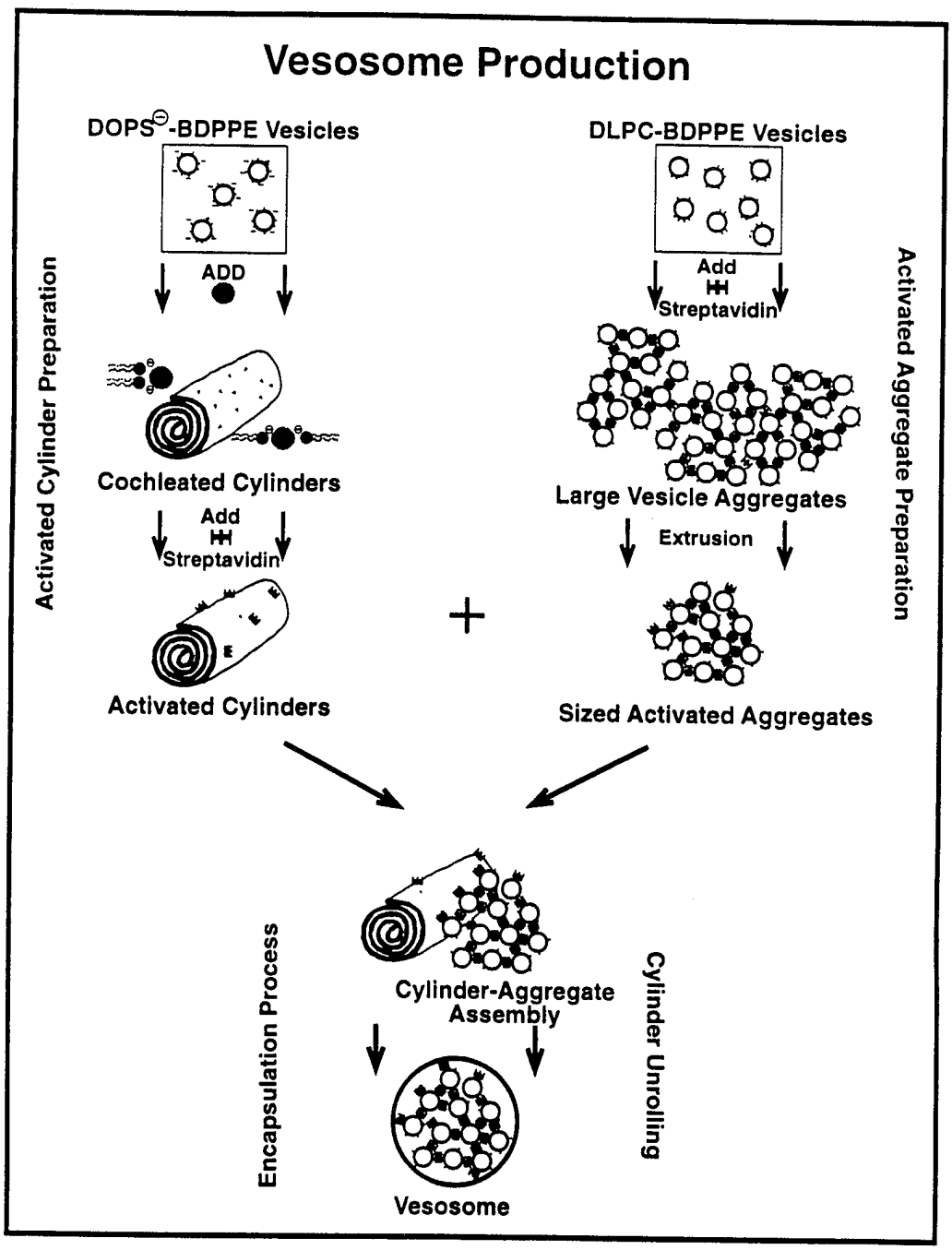

Vesosome Production
DOPS⊖-BDPPE Vesicles
ADD
Cochleated Cylinders
Add
Streptavidin
Activated Cylinders
Activated Cylinder Preparation
DLPC-BDPPE Vesicles
Add
Streptavidin
Large Vesicle Aggregates
Extrusion
Sized Activated Aggregates
Activated Aggregate Preparation
+
Cylinder-Aggregate Assembly
Encapsulation Process
Cylinder Unrolling
Vesosome

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21775

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127
US CL :424/450

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,618,560 A (BAR-SHALOM et al.) 08 April 1997, see column 6, line 44 - column 7, line 5, column 7, lines 18-20, column 13, lines 40-51 and Figure 4.	1-10 and 14-25.
A,P	US 5,643,574 A (GOULD-FOGERITE et al.) 01 July 1997.	1-25
A	US 5,492,696 A (PRICE et al.) 20 February 1996.	1-25
A	US 4,078,052 A (PAPAHADJOPOULOS) 07 March 1978.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 FEBRUARY 1998

Date of mailing of the international search report

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