Encapsulating Vesicles and Colloids from Cochleate Cylinders

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Encapsulating small vesicles or colloidal particles within a phospholipid bilayer affords multi-compartment structures that are interesting for targeted drug delivery, imaging, or separations. Dioleoylphosphatidylserine (DOPS), an anionic lipid, forms cochleate cylinders, tightly wrapped tubes of concentric bilayers, on addition of millimolar Ca²⁺. Complexation of Ca²⁺ by the subsequent addition of ethylenediaminetetraacetic acid (EDTA) results in the unwrapping of the cochleate cylinders of DOPS sheets and redosion to form micrometer-sized vesicles, which can entrap smaller vesicles in solution. Polystyrene spheres added to the cochleate cylinders before the EDTA unwrapping can also be enclosed.

Introduction

Nature utilizes phospholipid bilayers to segregate specific volumes or structures within cells to facilitate chemical or physical processes necessary for life. For example, the bilayer of a lysosome encapsulates a medium having the low pH necessary for intracellular digestion. Peroxisomes contain enzymes such as D-amino acid oxidase, urate oxidase, and catalase. The phospholipid bilayer of these organelles shields the rest of the cell from oxidase, urate oxidase, and catalase. The phospholipid composition can facilitate triggered release, as by hyperthermia. Low molecular weight hydrophilic drugs can be efficiently entrapped in the aqueous compartment of bilayer vesicles and can be targeted for in vivo delivery by incorporation of antibodies or destroy the contents. Colloidal particles can also be encapsulated via interdigitated bilayer phases, and magnetic nanoparticles have also been encapsulated in liposomes formed via reverse-phase evaporation for use as imaging agents or targeting of vesicle contents.

In our original attempt to create vesicles within vesicles, we relied on the specific recognition properties of streptavidin and biotinylated lipids to try to attach the vesicles to be encapsulated to cochleate cylinders of dioleoylphosphatidylserine.

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dyserine (DOPS). However, the efficiency of encapsulation was very low, and the streptavidin, in addition to being expensive, could lead to an immune response in vivo. Here we show that by eliminating the specific recognition steps, efficient encapsulation of vesicles and colloidal particles can be accomplished with cochleate cylinders (Figure 1). Vesicles of DOPS, an anionic lipid, fuse and form cochleate bilayer cylinders when Ca\(^{2+}\) is added. At this point, vesicles made from neutral lipids or colloidal particles not affected by Ca\(^{2+}\) can be added to the cylinders. Complexation of Ca\(^{2+}\) upon addition of EDTA affords unrolling of the cylinders and reclosure of the bilayers. The result is a nested assembly of DOPS liposomes and, if applicable, colloidal particles or vesicles of other phospholipids surrounded by a secondary, micrometer-sized envelope.

Materials and Methods

Materials. Dioleoylphosphatidylserine (>99%) (DOPS) in chloroform and dry dioleoylphosphocholine (>99%) (DSPC) and cholesterol (>98%) were used as received from Avanti Polar Lipids (Alabaster, AL). Stearylamine (98%) was used as received from Sigma (St. Louis, MO). Red fluorescent, carboxylate-modified polystyrene spheres (26 and 110 nm) were purchased from Molecular Probes (Eugene, OR). All other chemicals were from Aldrich (St. Louis, MO) and of the highest available purity. All suspensions of vesicles and solutions of Ca\(^{2+}\) and ethylenediaminetetraacetic acid (EDTA) were prepared in an aqueous buffer containing 10 mM N-[tris(hydroxymethyl)methyl]glycine (pH 7.4). EDTA, 0.1 mM EDTA, 0.2 g/L sodium azide (to inhibit bacterial growth), and 100 mM NaCl. The pH of the buffer was adjusted to 7.4 with HCl.

Preparation of 100 nm DOPS Vesicles. DOPS in chloroform was placed in a glass vial, and the solvent was evaporated under a stream of nitrogen. The lipid film was dried overnight under vacuum, and then sufficient buffer was added to bring the concentration to either 10 or 50 mg of lipid/mL. After being frozen in liquid nitrogen and thawed 10 times, the sample was extruded at room temperature at least 15 times through a polycarbonate Nucleopore filter having 100 nm pores (Corning Costar, Cambridge, MA).

Preparation of 200 nm Vesicles of DSPC/Cholesterol (DSPC/Cholesterol/Stearylamine). DSPC and cholesterol (and stearylamine) were dissolved in a 2/1 (2/1/1) molar ratio in chloroform in a glass vial, and then all solvent was evaporated under a stream of nitrogen. The lipid film was dried overnight under vacuum, and sufficient buffer was added to bring the total concentration of lipids to 100 mg/mL. After being frozen and thawed 10 times, the sample was sonicated at 70 °C for 20 min with a probe-tip sonicator (Fisher Scientific Sonic Dismembrator 600). Maximum output of the sonicator, 200 W, was used. The suspension of liposomes was then repeatedly extruded at 70 °C through a polycarbonate Nucleopore filter having 200 nm pores.

Encapsulation of DOPS Vesicles within an Outer Envelope of DOPS. An aliquot (0.1 mL) of a 50 mg/mL suspension of DOPS vesicles in buffer was placed in a vial. Increased turbidity upon the addition of 0.1 mL of a 12 mM solution of CaCl\(_2\) in buffer accompanied the fusion of DOPS vesicles and formation of cochleate cylinders. The sample was allowed to incubate overnight at room temperature. Formation of micrometer-sized envelopes of DOPS enclosing smaller vesicles was accomplished by adding 120 μL of a 20 mM solution of EDTA in buffer (400% excess) to each sample, vortexing, and incubating the samples overnight at room temperature. The DOPS vesicles formed spontaneously during the incubation period after EDTA addition.

Encapsulation of Neutral Vesicles within an Outer Envelope of DOPS. An aliquot (0.1 mL) of a 10 mg/mL suspension of DOPS vesicles in buffer was placed in a vial, and 0.2 mL of a 6 mM solution of CaCl\(_2\) in buffer was added. The sample was vortexed and incubated overnight at room temperature to form cochleate cylinders as above. An aliquot (0.1 mL) of a suspension of 2/1 DSPC/cholesterol or 2/1/1 DSPC/cholesterol/stearylamine vesicles in buffer (see above) was added to the sample. The sample was vortexed. An aliquot of 0.1 mL was withdrawn, and to this aliquot was added 60 μL of a 10 mM solution of EDTA (400% excess) in buffer. The sample was vortexed and incubated at room temperature for 6 days before freeze–fracture replicas were prepared.

Encapsulation of 26 nm Polystyrene Spheres within DOPS. An aliquot (0.1 mL) of a 50 mg/mL suspension of DOPS vesicles in buffer was placed in a vial, and 0.2 mL of a 12 mM solution of CaCl\(_2\) in buffer was added to form cochleate cylinders. The sample was vortexed and incubated overnight at room temperature. The suspension of microspheres as received (4.5 x 10\(^{10}\) mL\(^{-1}\)) was sonicated for 30 min, and then 0.1 mL was added to the prepared cochleate cylinders of DOPS and vortexed. An aliquot of 0.2 mL was withdrawn, and to this aliquot was added 120 μL of a 20 mM solution of EDTA in buffer (400% excess). The sample was vortexed and incubated at room temperature for 3 h before freeze–fracture replica was prepared.

Encapsulation of 110 nm Polystyrene Spheres within DOPS. An aliquot (0.1 mL) of a 10 mg/mL suspension of DOPS vesicles in buffer was placed in an Eppendorf vial, and 0.2 mL of a 0.1mL of a 20 mM solution of CaCl\(_2\) in buffer was added. The sample was vortexed and incubated overnight at room temperature. The suspension of microspheres as received (4.5 x 10\(^{10}\) mL\(^{-1}\)) was sonicated for 30 min, and then 0.1 mL was added to the prepared cochleate cylinders of DOPS and vortexed. An aliquot of 0.2 mL was withdrawn, and to this aliquot was added 120 μL of a 20 mM solution of EDTA in buffer (400% excess). The sample was vortexed and incubated at room temperature for 3 h before freeze–fracture replica was prepared.
several days in tetrahydrofuran. The replicas were collected on spheres, the replicas were subsequently allowed to stand for any remaining phospholipid. If the sample contained polystyrene and warmed to room temperature. The copper planchettes were to the surface. The replicas were then removed from the vacuum at a 45° angle, followed by about 15 nm of carbon deposited normal to the surface. The replicas and formation of tightly wrapped, multilamellar, cochleate cylinders of DOPS bilayers (Figure 2a). When the original concentration of 100 nm vesicles was high (50 vs 10 mg/mL), a correspondingly higher concentration of unfused vesicles remained after the formation of cylinders. These unfused vesicles often aggregated and could be removed from the cylindrical assemblies by centrifuging. When no separation was done, and an excess of EDTA was added to complex the Ca$^{2+}$, the cylinders unwound. Unfavorable edge energy was eliminated by closure of the sheets back into spheres, entrapping the unfused DOPS vesicles by an outer DOPS bilayer (Figure 2b). The wide size range of the interior vesicles revealed that some had partially fused, since they were generally larger than the starting 100 nm vesicles. The diameter of the exterior envelope, which was typically unilamellar, was on the order of micrometers. While the size of these assemblies as prepared may restrict their application to depot drug delivery vehicles, extrusion through filters of defined pore size may yield smaller, multicompartamental structures more amenable to parenteral delivery.9,27,28

Encapsulation of Neutral Vesicles. Multicompartamental vesicle assemblies also formed when 200 nm neutral vesicles of diesteroylphosphatidylcholine (DSPC) and cholesterol (2/1 mol/mol, respectively) were added to cochleate cylinders of DOPS prior to complexation of Ca$^{2+}$ by EDTA. In an effort to optimize encapsulation, we examined the effects of several parameters, including the concentration of DOPS. Also, the neutral liposomes were added to either the 100 nm DOPS vesicles (before Ca$^{2+}$) or the cochleate cylinders of DOPS (after Ca$^{2+}$). No one set of conditions was clearly better than another; all replicas reveal cross-fractured, micrometer-sized vesicles that contain smaller interior vesicles (Figure 3a). A sample that tended to display interior liposomes larger than 200 nm (i.e., fusion-produced interior liposomes) was prepared by using a relatively high DOPS concentration (12.5 mg/mL immediately before the addition of EDTA) and by adding the neutral vesicles to the preformed, Ca$^{2+}$-induced cylinders of the anionic phospholipid. All other conditions afforded encapsulation of a significant population of 200 nm vesicles of 2/1 DSPC/cholesterol. When the neutral vesicles were added before Ca$^{2+}$, some of the liposomes were trapped as the cochleate cylinders formed and, after the addition of EDTA, were isolated in pockets between layers of a micrometer-sized, multilamellar structure.

The process of creating encapsulated assemblies of vesicles is very efficient; all large structures that cross-fractured contained smaller liposomes. However, a fraction of the small vesicles remained unenclosed by a secondary bilayer regardless of whether they were DOPS or 2/1 DSPC/cholesterol (Figures 2b and 3a, respectively). As a result, the vesicle size distribution is bimodal, with significant populations of both large and small vesicles (Figure 3b). The micrometer-sized vesicles are formed in a sufficient concentration that this is a viable method for efficiently trapping small vesicles or other materials. Closely packed spherical structures such as the DOPS exterior membranes can only occupy up to 60–70% of the solution volume due to packing constraints; it is likely that the maximum entrapment efficiency is also of this magnitude.

To develop this method of forming liposomes for potential applications, further investigations will involve the separation of encapsulated from unencapsulated material. We and other research groups have accomplished similar size-based separations of other kinds of liposomes

Figure 2. Freeze-fracture TEM micrographs of intermediate (a) and final (b) structures in the encapsulation of phospholipid vesicles within an outer envelope of distearoylphosphatidylserine (DOPS). (a) Cochleate cylinders of DOPS (parallel to white arrows). The cylinders were formed when 100 nm anionic DOPS vesicles (50 mg of lipid/mL, 0.1 mL) fused upon the addition of Ca$^{2+}$ (0.1 mL of 12 mM CaCl$_2$). Although some unfused 100 nm vesicles of DOPS are still present (for example, at the black arrows), the cylinders could be isolated by centrifugation or chromatography. (b) When a 400% excess of EDTA was added to Ca$^{2+}$-induced DOPS cylinders, an assembly of vesicles with unvesicles formed. The outer envelope of DOPS (white arrow), more than a micrometer in diameter, encloses smaller vesicles of the same phospholipid (for example, at the black arrows). of a 6 mM solution of CaCl$_2$ in buffer was added with mixing. Following incubation overnight at room temperature, the cochleate cylinders were pelleted by centrifuging at 1000 g for 5 min, and then the supernatant was removed. Before their addition to the DOPS cylinders, the polystyrene spheres were concentrated by centrifuging 0.5 mL of the suspension as received (2.7 x 10$^{13}$ mL$^{-1}$) in a 100 000 molecular weight cutoff centrifuge filter (Millipore) at 21 000 g for 10 min, followed by adjusting the volume to 100 µL with deionized water. To the DOPS cylinders were added 40 µL of this concentrated suspension, followed by 40 µL of 120 mM EDTA in buffer (800% excess relative to the original amount of Ca$^{2+}$ added). The sample was vortexed and incubated at room temperature overnight before freeze-fracture replicas were prepared.

Preparation of Replicas for Electron Microscopy. Standard procedures were used to prepare platinum-carbon replicas for electron microscopy.24 A film of sample approximately 100 µm thick was trapped between two copper planchettes and frozen by plunging it into a liquid propane/liquid ethane bath cooled by liquid nitrogen. The frozen sample was transferred under liquid nitrogen to the sample block of a JE EM Cryofract freeze-fracture device. After specimen loading, the block was quickly transferred to the vacuum chamber of the Cryofract device via an airlock. After temperature (~170 °C) and pressure (~10$^{-7}$ Torr) equilibration, the sample was fractured and the two resulting surfaces were replicated with approximately 1.5 nm platinum deposited at a 45° angle, followed by about 15 nm of carbon deposited normal to the surface. The replicas were then removed from the vacuum and warmed to room temperature. The copper planchettes were dissolved in chromomer, and then the replicas were washed in water and allowed to stand in ethanol for several days to dissolve any remaining phospholipid. If the sample contained polystyrene spheres, the replicas were subsequently allowed to stand for several days in tetrahydrofuran. The replicas were collected on Formvar-coated TEM grids. A Gatan CCD camera was used to record digital bright-field images from the 100CXII transmission electron microscope.

Results and Discussion

Encapsulation of DOPS Vesicles with DOPS Bilayers. When Ca$^{2+}$ was added to 100 nm diameter vesicles of DOPS, the turbidity increased, a sign of the fusion of the vesicles and formation of tightly wrapped, multilamellar, cochleate cylinders of DOPS bilayers (Figure 2a).22


Other researchers have already examined the perme-ability of unilamellar phosphatidylserine (PS) liposomes.

Encapsulation of Colloidal Particles. Like phos-pholipid vesicles, colloidal particles can also be enclosed within a bilayer of DOPS via Ca\(^{2+}\)-induced cylinders of the anionic lipid. To illustrate this capability, we encapsulated carboxylate-modified polystyrene spheres (Figure 4). Passive encapsulation yielded structures in which the density of spheres was the same inside and outside the DOPS bilayer. Any unenclosed particles could potentially be separated from the vesicles by centrifugation or size-exclusion chromatography.

Conclusion

Defined regions of space, with delineated boundaries and controllable mechanisms for entrance and exit, are essential to making all but the most primitive functional nanostructures. Nature has solved this problem by using lipid bilayer membranes to divide cells into a distinct inside and outside, and the properties of the bilayer cause chemical species to cross this barrier at widely different rates.
rates. Moreover, nature typically relies on a nested series of membranes to perform complex tasks. A set of nested bilayers should provide a degree of freedom for optimization not possible with a single membrane and a more realistic approximation of biological organization. However, it has proven difficult to encapsulate anything larger than molecular solutions within lipid bilayers by conventional vesicle self-assembly. Encapsulating vesicles or other sensitive materials within a bilayer is complicated by the need to protect the interior contents while the exterior membrane is forming, which eliminates most conventional vesicle-forming processes. What is necessary is to have a metastable phase of bilayer sheets that can be opened and closed by processes that do not disrupt other vesicles or compromise biologically or chemically sensitive materials.

Dioleoylphosphatidylserine (DOPS), an anionic lipid, forms tightly wrapped, cylindrical bilayers known as cochleate cylinders when Ca\(^{2+}\) is added.\(^{22}\) Subsequent addition of EDTA causes the cylinders to unravel and form micrometer-sized liposomes. During the reversion, the DOPS bilayers can entrap DOPS vesicles, neutral vesicles, and colloidal particles with good efficiency. This method is highly efficient for the formation of nested structures; every micrometer-sized vesicle that cross-fractured contained smaller vesicles or colloidal particles.

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