CONTROLLED MULTI-STAGE SELF-ASSEMBLY OF VESICLES

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ABSTRACT

The association of lipid or surfactant molecules into spherical vesicles in solution constitutes a primary self-assembly process, although typical vesicles are not the equilibrium form of aggregation for most lipids. Such meta-stable vesicles can undergo a secondary self-assembly into higher order structures in a controlled and reversible manner by means of site-specific ligand-receptor coupling. Cryo-electron microscopy shows these structures to be composed of tethered vesicles in their original, unstrained state. In contrast, vesicles aggregated by non-specific forces are deformed. In this work, we show that equilibrium vesicles can also undergo a secondary self-assembly via ligand-receptor interactions, as evidenced by freeze-fracture electron microscopy. Such site-specific vesicle aggregation provides a practical mechanism for the production of stable, yet controllable, microstructured materials.

INTRODUCTION

Liposomes are spherically enclosed bilayer structures which separate an aqueous interior from an aqueous exterior. In vivo, liposomes can exist as unilamellar vesicles (ULVs), single-bilayer closed shells. However, in vitro, the equilibrium structure of pure phospholipid liposomes in excess water is as multilamellar vesicles (MLVs). MLVs have many concentric bilayers separated by thin layers of water. Producing ULVs from phospholipid dispersions results in the formation of meta-stable, non-equilibrium structures that eventually revert back to their equilibrium, multilamellar structures.

Equilibrium unilamellar vesicle structures have been observed to form spontaneously only in mixed surfactant systems. Evans and coworkers found equilibrium ULVs in solutions of didodecyldimethylammonium surfactants with a range of counterions. Murthy et al. discovered equilibrium vesicles in mixtures of the surfactant Aerosol OT and choline counterions. Gebicki and Hicks reported spontaneous vesicle formation in mixtures of fully ionized and unionized oleic and linoleic acids at pH 8-9. Jain et al. have reported spontaneous formation of ULVs and MLVs in mixtures of lyssolecithins and fatty acids. Hauser has shown spontaneous vesicles form in mixtures of lysolecithin and lecithin. Kaler et al. have demonstrated the existence of spontaneous, monodisperse, equilibrium vesicles in aqueous mixtures of cetyl trimethylammonium tosylate (CTAT), a cationic surfactant, and sodium dodecylbenzene sulfonate (SDS), an anionic surfactant. In this "catanionic" system, the oppositely charged surfactants form a type of neutral dimer complex that resembles a double-tailed phospholipid and is thus able to form bilayers.

Unilamellar vesicles are the preferred structure for many applications due to their higher encapsulation efficiency compared to MLVs. Vesicles are also being used extensively as model biomembranes; since cell-membranes are typically composed of 60-70% phospholipid molecules, understanding the physical properties of pure phospholipid vesicles can provide insight into the properties of cells. Vesicles have been proposed for use as site-specific drug delivery vehicles, in which an aqueous solute (e.g. drug) is encapsulated into the aqueous interior of the vesicle and induced to interact with an (infected) cell through adhesion, fusion, or endocytosis with the cell membrane. Vesicles have also been used as microreactors for synthesizing new materials, and are already being used in the cosmetics industry. Vesicles have been proposed for use as affinity chromatography agents, in which vesicles are coated with a small fraction of ligands that specifically bind to a desired protein, allowing the vesicle to separate the desired protein from a sea of many proteins. The vesicles can then be concentrated by dialysis or centrifugation.

Not only do individual vesicles have potential applications, there exist possible applications for vesicle aggregate structures, from bioengineered artificial tissues to advanced drug delivery systems. In this work, we outline a way to use the properties of catanionic surfactants and ligand-receptor interactions to go directly from molecular solution to macroscopic organization.
MATERIALS AND METHODS

Materials

Dilauroylphosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Cetyltrimethylammonium tosylate (CTAT), obtained from Alfa Chemicals (Danvers, MA), was recrystallized from a 1:1 mixture of ethanol and acetone. Branched sodium dodecylbenzene sulfonate (SDBS) was used as received from Tokyo Kasei (Japan). Biotin-X-Dipalmitoylphosphatidylethanolamine (B-DPPE) and streptavidin were obtained from Molecular Probes (Eugene, Oregon). All aqueous dispersions of DLPC/B-DPPE and CTAT/SDBS/B-DPPE were prepared in Milli-Q Plus (Millipore Co., Bedford, MA) deionized water.

Sample Preparation

DLPC and B-DPPE were dissolved in chloroform and the solvent was subsequently removed thoroughly under vacuum. The mixed lipids were hydrated in an aqueous 0.2 M NaCl and 0.2 mM phosphate buffer to form a multilamellar vesicle dispersion, vortexed gently, hydrated for approximately 48 hours at 40°C, and subsequently equilibrated for several days at 25°C. The dispersion was repeatedly frozen in liquid nitrogen and thawed, followed by repeated extrusions through stacked Nucleopore filters of specific pore size 100 nm, to form a monodisperse population of unilamellar vesicles. CTAT, SDBS, and B-DPPE were dissolved in chloroform and the solvent was removed under vacuum. The mixed surfactants were hydrated in an aqueous dispersion of 0.05 M NaCl, forming spontaneous equilibrium vesicles with biotin moieties protruding from the vesicles. Streptavidin was added to either solution to induce aggregation.

Cryo-Electron Microscopy

Samples for cryo-Transmission electron microscopy (Cryo-TEM) were prepared in an environmental chamber at >95% humidity at 25°C. The sample was vitrified by quick plunging into liquid propane or ethane cooled by a surrounding bath of liquid nitrogen. The vitrified specimens were transferred under liquid nitrogen and mounted onto a Gatan 626 cold-stage transfer module (Gatan, Inc., PA). The sample holder temperature was maintained below -165°C during imaging with a JEOL 2000FX electron microscope operated at 100 kV in the conventional TEM mode.

Freeze-Fracture Electron Microscopy

Samples for freeze-fracture transmission electron microscopy (FF-TEM) were prepared by placing ~0.5 μL of sample between two thin (0.1 mm) copper freeze-fracture planchettes (Balzers Union, BU-012-056T, Hudson, NH). Rapid freezing was done as described above. The vitrified specimens were transferred under liquid nitrogen to a Balzers 400 freeze etch device, fractured under vacuum (<10⁻⁷ mbar) at -170°C and then immediately shadowed with a 1.5 nm thick layer of platinum deposited at a 45° angle with respect to the fracture surface, followed by a 20 nm thick layer of carbon deposited normal to the surface to create a permanent replica of the fractured surface. The replicas were cleaned according to standard procedures and imaged with a JEOL 100CX II electron microscope in the conventional transmission mode at 100 kV.

RESULTS AND DISCUSSION

Cryo-TEM of a 100 mg/ml phosphatidylcholine (PC) aqueous dispersion (Fig. 1A) reveals spherical, unstressed ULVs. Vesicles such as this can be induced to aggregate by stressing them osmotically, as observed by FF-TEM of similarly prepared PC vesicles (Fig. 1B). Osmotic stress suppresses the repulsive undulation force and enhances the attractive hydrophobic force between them. The contact region between the aggregated vesicles is flat, a result of nonspecific surface forces that exist over the entire vesicle bilayer, which attempt to minimize exposure of the hydrophobic tails to the water. Deformations produced by such aggregation may cause large elastic strains and stresses on the bilayers, resulting in increased leakage, lysis, and fusion.
Figure 1. (A) Cryo-electron micrograph of freely floating PC vesicles. (B) Freeze-fracture electron micrograph of osmotically stressed PC vesicles undergoing non-specific aggregation.

Such deformations are minimized when vesicles are coupled by specific interactions. The whole vesicle surface is not involved in the interaction, only a discrete number of highly localized biotin-streptavidin pairs. Biotin is a small, water-soluble molecule that specifically binds to one of four binding sites (including two on opposite sides of the molecule, allowing for crosslinking of two biotin surfaces) on streptavidin, a large, water-soluble protein molecule. One interesting feature of this interaction is the extremely high binding energy per streptavidin binding site, about 30-35 kT. What renders this system so versatile is the fact that biotin can be conjugated to the headgroup of a phospholipid (e.g. DPPE) while (1) the biotin headgroup maintains its ability to bind to the streptavidin binding site and (2) the phospholipid maintains its ability to be incorporated into bilayers. However, mainly due to steric effects, lipid-conjugated biotin has a lower binding energy with streptavidin, about 15-16 kT per bond.

To test the hypothesis that site-specific binding induced aggregation does not deform vesicles, we first prepared 100 nm ULVs of DLPC and B-DPPE at 30 mg/ml total lipid concentration and 0.17 mol% B-DPPE in an aqueous solution of 0.2 M NaCl and 0.2 mM phosphate buffer by extrusion. Streptavidin was then added to the ULV dispersion (at a 7:1 ligand-receptor mole ratio) to produce a final lipid concentration of 20 mg/ml. The vesicles appeared to flocculate immediately, as the appearance of the solution changed from initially clear blue (ULV dispersion) to cloudy white (aggregated vesicles). Figure 2A is a schematic representing the geometry of the site-specific aggregation of vesicles induced by adding streptavidin to the DLPC/B-DPPE vesicle dispersion. At the concentration used, approximately 80 B-DPPE ligands were exposed on the outer monolayer of the vesicles. Figure 2B is a cryo-electron micrograph of the vesicle aggregates approximately 30 minutes after streptavidin addition. The arrows indicate specific points of contact between aggregated vesicles. The vesicles appear to remain spherical, indicating that the vesicles remained unstressed during aggregation. Virtually all of the vesicles were present in large aggregates; few free vesicles were seen in the micrographs. In addition, the aggregates seem to be bound together strongly enough that they did not break up, even during the relatively large shears necessary to form thin samples for Cryo-TEM. No such aggregates were seen in control experiments where (1) streptavidin was added to a solution of pure DLPC vesicles and (2) streptavidin inactivated by soluble biotin binding-site saturation was added to the DLPC/B-DPPE solution.
The aggregation of colloidal particles often times can be reversed. However, due to the high binding affinity of the ligand-receptor bond used in our system, we initially thought that aggregation would be irreversible. Fortunately, ligand-receptor bonds can be reversed by the addition of an even higher affinity agent to the solution, one that effectively competes with and displaces the originally bound ligand. To demonstrate reversibility in our system, we added 10-fold excess of soluble biotin to streptavidin to the aggregated vesicles. Soluble biotin has a much higher binding energy (and thus higher affinity) than the biotin moiety on B-DPPE, as mentioned previously. Approximately 30 minutes after soluble biotin addition, the vesicles in the aggregates had redispersed back to their freely floating, spherical, unstressed shape, as indicated by Cryo-TEM (not shown). Hence, this vesicle aggregation can be switched on and off controllably.

One important consideration in the DLPC/B-DPPE plus streptavidin vesicle aggregation process is that the actual vesicles used were non-equilibrium in nature; they were forced into ULVs by extrusion of an equilibrium MLV dispersion. Thus, these vesicles may eventually revert back to their equilibrium structure, possibly destroying the aggregate network in the process. It is therefore of interest to produce an equilibrium vesicle aggregate structure. If the vesicles are themselves equilibrium structures, then the aggregates most likely will be also.

As described earlier, one system that produces equilibrium unilamellar vesicles are mixtures of cationic and anionic surfactants (catanionic vesicles), most notably CTAT and SDBS. To determine whether or not cationic vesicles will self-assemble into larger, higher-order aggregates, we first prepared two aqueous 0.05 M salt solutions consisting of CTAT, SDBS, and B-DPPE at a total surfactant concentration of 2.0 wt%, CTAT/SDBS weight ratio of 4/1 and 1/4, respectively, with a B-DPPE concentration of approximately 0.1 mol %. Equilibrium unilamellar vesicles are reported to form at the concentrations and ratios of CTAT and SDBS described. However, incorporation of the third component, B-DPPE, led to some question as to whether unilamellar vesicles would indeed form. Figure 3A is a freeze-fracture electron micrograph of the 2.0 wt%, 1/4 CTAT/SDBS plus B-DPPE aqueous dispersion, several days after mixing (to allow the system to equilibrate). Note that unilamellar vesicles do indeed form, and the vesicles are spherical and are not aggregated. At the B-DPPE concentration used, approximately 100 biotin ligands are exposed on the outer monolayer of the average CTAT rich vesicle, and approximately 35 for the average SDBS rich vesicle. Addition of streptavidin to these solutions (at a ligand-receptor ratio of 8:1, diluting the surfactant concentration to 1.9 wt%) resulted in limited

Figure 2. (A) Schematic illustration and (B) cryo-electron micrograph of DLPC vesicles aggregating by site-specific biotin-streptavidin coupling. Molecule is B-DPPE.

Figure 3. (/) vesicles prior to aggregation u
aggregation of the vesicles. Figure 3B is a freeze-fracture electron micrograph of the 2.0 wt%, 1/4 CTA7/SDBS and B-DPPE solution approximately 30 minutes after streptavidin addition, showing that vesicles are indeed aggregated, but not to the extent that appears in the cryo-electron micrographs of the DLPC system. There seem to exist several vesicles that have not aggregated. However, as the arrows indicate, there are large aggregates of vesicles that also appear to be spherical and unstressed. Subsequent freeze-fracture experiments on samples taken five days later show no apparent increase in vesicle aggregation (not shown). As the degree of aggregation was not as high as in the DLPC experiments, no reversibility experiments were performed, but presumably, adding excess soluble biotin would again completely reverse the aggregation.

Figure 3. (A) Freeze-fracture electron micrograph of 2.0 wt%, 1/4 CTA7/SDBS plus B-DPPE vesicles prior to streptavidin addition. (B) Micrograph of vesicles of same system which have aggregated upon addition of streptavidin.
Although cryo-electron microscopy appears to be better suited for imaging such large vesicle aggregates, it is not likely the appearance of a higher degree of aggregation in the DLPC experiments is a consequence of the chosen microscopy technique; freeze-fracture experiments on the DLPC aggregates (not shown) indicate that there is indeed a higher degree of aggregation than in the CTAT/SDBS aggregates. The most likely reason equilibrium vesicle aggregation is much lower is due to the fact that the catanionic vesicles are charged, and hence, exhibit long-range electrostatic repulsion. However, this work shows it is possible to go directly from molecular solutions to multi-vesicular aggregates by simple, spontaneous self-assembly.

References


