Multiple Lipid Compartments Slow Vesicle Contents Release in Lipases and Serum

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ABSTRACT Unilamellar vesicles or “liposomes” are commonly used as simple cell models and as drug delivery vehicles. A major limitation of unilamellar liposomes in these applications has been premature contents release in physiological environments. This premature release is likely due to enzyme degradation or protein insertion into the liposome membrane, which significantly increases the bilayer permeability. Encapsulating unilamellar liposomes within a second bilayer to form multicompartiment “vesosomes” extends contents retention by 2 orders of magnitude by preventing enzymes and/or proteins from reaching the interior bilayers. The multicompartiment structure of the vesosome can also allow for independent optimization of the interior compartments and exterior bilayer; however, just the bilayer-within-a-bilayer structure of the vesosome is sufficient to increase drug retention from minutes to hours. The vesosome is a better mimic of eukaryotic cell structure and demonstrates the benefits of multiple internal bilayer-enclosed compartments.

KEYWORDS: liposomes · vesicles · serum · phospholipase · bilayers

Conventional unilamellar vesicles used in drug delivery and as cell models have a single compartment delimited by a single bilayer membrane, which mimics the single-compartment structure of the prokaryote cell.¹ This bilayer defines the interior space, regulates release of the vesicle contents, and protects the vesicle contents from degradation.² Essentially all of the literature on vesicles and liposomes involves modifying the chemical and physical properties of this single bilayer to optimize these tasks.^{1,3–7} Despite 30 years of extensive research, only doxorubicin (or the chemically similar daunorubicin) for chemotherapy⁸ and amphotericin B in anti-fungal preparations are currently clinically available in liposome formulations.¹ Many other drugs, such as the antibiotic ciprofloxacin and the chemotherapy agents vinorelbine, vinblastine, and vincristine, leak out too rapidly from liposomes in a physiological environment,^{9–15} which severely limits the efficacy of unilamellar vesicles as drug carriers. While coating the exterior of vesicles with lipids functionalized with poly(ethylene glycol) (PEG) shields

against immune recognition^{1,16} and extends *in vivo* circulation times to 24–48 h, PEG-lipids actually increase permeability and do not enhance drug retention.^{17,18}

Maximizing vesicle contents retention has led to a variety of choices of lipid head-group, saturation of the lipid alkyl chains, and the addition of cholesterol. Many of these modifications decrease bilayer permeability by orders of magnitude *in saline*; however, the differences in contents retention with bilayer composition are less pronounced in serum and other relevant physiological environments.^{9,10,13–16,19} This suggests that vesicle contents release is due to degradation of the liposome bilayer by lipases, complement, or other enzymatic processes, rather than by simple permeation.^{1,16}

Slowing drug release is essential because extending the time of vesicles in the circulation results in accumulation at tumors or inflammation sites due to the mechanism known as the enhanced permeability and retention (EPR) effect.¹ Rapidly growing or remodeling blood vessels near tumors, unlike those in most normal tissues, have gaps as large as 600–800 nm between adjacent endothelial cells through which drug carriers can extravasate.¹ In addition, because tumors have impaired lymphatic drainage, the carriers concentrate in the tumor, and large increases in tumor drug concentrations could be achieved if the drug is successfully retained in the carrier.¹ The benefits of the 24–48 h circulation time of liposomes coated with PEG-lipids^{1,16} are lost if the liposome contents leak out over shorter periods of time.

A novel solution to slowing drug release is to encapsulate unilamellar liposomes within another liposome, similar to

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the structure of the eukaryote cell. Eukaryotes developed this structure as a successful alternative to optimizing the chemistry and physics of a single bilayer. The use of multiple, nested compartments, each with a distinct bilayer membrane, has proven to be a successful way to separate different cellular functions while protecting important internal contents from the environment. Mimicking these nested bilayer compartments led to the development of a multicompartment structure of unilamellar vesicles trapped within an exterior membrane called the “vesosome” (Figure 1).^{20–22} The inner compartments of the vesosome can encapsulate multiple drugs (to deliver drug cocktails²³) or have different bilayer compositions to optimize release. In addition, while it is difficult to encapsulate anything larger than molecular solutions by conventional vesicle self-assembly, the vesosome construction process lends itself to trapping colloidal particles and biological macromolecules.^{20–22,24} We show here that the nested bilayer compartments of the vesosome provide a degree of freedom for optimization not possible with a single compartment and are a more realistic approximation of higher order biological organization. The vesosome extends drug retention during exposure to phospholipase A₂ by orders of magnitude compared to unilamellar vesicles of the same composition. The exterior membrane provides a physical barrier against the lipase, which prevents the lipase from degrading the interior compartments. The exterior membrane also protects the interior vesicles from binding to streptavidin when the interior lipid bilayers are decorated with biotinylated lipids. We extend these results to the more complex environment of serum to show that model drug release is slowed by 2 orders of magnitude compared to that of unilamellar vesicles. The physical barrier of the external membrane is extremely effective in protecting the interior compartments from degradation, thereby enhancing drug retention in physiological environments.

RESULTS

To make vesosomes (or to encapsulate other colloidal suspensions), it is necessary to encapsulate vesicles efficiently, without damage, within a different membrane. In practical terms, open bilayer sheets must be formed which can then be closed without compromising the drug-carrying lipid vesicles to be encapsulated. For this purpose, we take advantage of the reversible, ethanol-induced interdigitation of saturated phosphatidylcholine bilayers. Below the main transition temperature, T_m , a number of saturated phospholipids form an $L_{\beta 1}$ phase on addition of ethanol in which the all-trans acyl chains of the opposing monolayers interdigitate.^{21,24,25} This interdigitation increases the membrane rigidity, and the increased curvature energy leads to the formation of bilayer sheets^{21,24} rather than closed vesicles. Heating above T_m causes the acyl chains

Figure 1. (A) Freeze-fracture TEM images of as-formed vesosome. Vesosomes consist of a single or multiple membrane shell surrounding an aqueous core that contains small (here 50 nm) unilamellar vesicles as internal compartments. The interior density of the 50 nm vesicles (arrows) is the same as that outside the vesosome. (B) After one sedimentation step and two negative selections with streptavidin-coated magnetic beads, there are no small vesicles outside the vesosome, but the interior density of the vesicles is the same as in panel A. Freeze-fracture samples were prepared by standard techniques.^{32–36}

to melt, and the membrane reverts to a disordered, L_{α} bilayer phase. The reduced bending rigidity of the L_{α} phase allows the bilayers to close; in the process, small vesicles and colloidal particles—essentially anything of nanometer dimensions in the original suspension—can be encapsulated,²¹ as shown in Figure 1.

TABLE 1. Vesicle Encapsulation Fraction for Different Vesicle Concentrations in the Original Suspension^a

vesicle concn (mg/mL)	vesicle encapsulation fraction (%)		
	raw vesosomes	1 sedimentation	1 sedimentation + 2 Biomag
100	53.1 ± 7.1	89.0 ± 3.8	99.0 ± 0.6
50	62.2 ± 3.2	87.5 ± 1.8	99.0 ± 0.5

^aA 0.5 mL portion of DPPC 50 nm vesicles (labeled with 0.02 mol % b-BODIPY 581/591 C₅-HPC, 0.1 mol % DHPE Biotin-X) at different concentrations was mixed with 25 mg of interdigitated DPPC bilayers and heated at 46 °C for 20 min to form vesosomes. Separation of unencapsulated vesicles from the vesosomes was carried out by one sedimentation step, refined by two BioMag negative selections in which the biotinylated vesicles were bound to streptavidin-coated magnetic particles and physically removed from the suspension with an external magnet.

The fraction of biotinylated vesicles encapsulated in vesosomes was determined by incubating the vesosomes with BODIPY-labeled avidin. The green fluorescence intensity of the BODIPY-labeled avidin ($\lambda_{\text{ex}} = 504$ nm, $\lambda_{\text{em}} = 512$ nm) is linearly proportional to the number of biotin-lipids bound; this allows the direct quantification of the biotin-lipids accessible to the avidin on the unencapsulated vesicles.^{26,27} The total amount of lipid in the vesicles in the suspension was determined after micellization with reduced Triton X-100 (RTX-100), by measuring the red fluorescence of the b-BODIPY 581/591 C₅-HPC incorporated in the bilayers of the vesicles ($\lambda_{\text{ex}} = 582$ nm, $\lambda_{\text{em}} = 593$ nm) against calibration samples.

Table 1 shows that vesosome formation results in 58% of the biotinylated vesicles not binding to the fluorescently labeled avidin. These vesicles were encapsulated and effectively shielded from binding to avidin by the exterior bilayer of the vesosome. This encapsulated fraction is reasonable in that a random packing of monodisperse spheres occupies a volume fraction of 56–64%, depending on the details of the packing;²⁸ polydispersity increases the encapsulated volume fraction. Encapsulation *via* the vesosome process is passive: the fraction of vesicles encapsulated is similar to the expected volume fraction of the random packing of polydisperse spheres (Figure 1). Our results show that avidin, which is a 66 kDa protein, cannot cross the exterior vesosome membrane; the absence of binding to 58% of the biotinylated vesicles also confirms that the interior vesicle bilayers do not fuse with the exterior vesosome membrane over the course of the experiment. Figure 1A shows transmission electron microscopy (TEM) images of the as-formed vesosome suspension; a roughly equal density of small vesicles is trapped within the vesosome as outside the vesosome. The TEM images also show that the interior vesicles are distinct and do not fuse with the exterior membrane.^{21,24,29}

While a mixed population of vesicles and vesosomes might be useful in certain applications, to distinguish release properties requires the quantitative separation of the unencapsulated vesicles from the

vesosome suspension. This (Table 1) is achieved by diluting the vesosome suspension, typically 1 mL in volume, in 125 mL of buffer and allowing the vesosomes to sediment overnight, followed by collection of the sedimented material. The method takes advantage of the size difference between the vesosomes, which have an average diameter of about 1 μm , and the unencapsulated 50 nm vesicles. After a single sedimentation step, the encapsulated fraction increases to 88% (Table 1).

If required, the separation can be refined by a second sedimentation step or a negative selection procedure that also takes advantage of the barrier properties of the vesosome exterior membrane. Mixing the once-refined vesosome suspension with streptavidin-coated magnetic particles causes any unencapsulated, biotinylated vesicles to bind to the magnetic particles. The magnetic particles are then physically separated from the suspension with an external magnet, leaving behind the purified vesosome suspension for collection. After two negative selection steps, no fluorescence increase relative to the biotin-free control was observed on addition of BODIPY-labeled avidin; hence, there were no detectable unencapsulated vesicles in the purified vesosome solution. Freeze-fracture TEM images (Figure 1B) confirmed the absence of unencapsulated vesicles in the suspension²⁹ while showing that the vesosomes retained the interior vesicles.

Vesosome and Vesicle Contents Release. The external membrane that protects the biotinylated lipids on the interior compartments from binding to the streptavidin on the magnetic particles also protects the interior contents of the vesosomes from active degradation by lipases. To quantify this protective effect, carboxyfluorescein (CF) release from unilamellar vesicles and that from identical vesicles encapsulated within a vesosome of the same membrane composition are compared in Figure 2. DPPC vesicles were formed by extrusion in 20 mM CF, and the external CF was quantitatively removed by size exclusion chromatography. A fraction of these CF-containing vesicles was then encapsulated within a DPPC membrane to form vesosomes and purified by repeated sedimentation as described earlier. At the concentration initially present in the vesicles, CF fluorescence is quenched; however, any released CF is diluted in the external solution, and the fluorescence is proportional to the CF solution concentration. Measuring the solution fluorescence with time gives the release profile: % release = $(I(t) - I(0))/(I_{\text{tot}} - I(0))$, where $I(t)$ is the fluorescence intensity at $\lambda_{\text{ex}} = 492\text{nm}$, $\lambda_{\text{em}} = 517\text{nm}$, at time t after exposure to the lipase, I_{tot} is the sample total fluorescence as determined after micellization by RTX-100, and $I(0)$ is the solution fluorescence at time zero.

Phospholipase A₂ (porcine pancreatic PLA₂, Sigma, St. Louis, MO) catalyzes the hydrolysis of the ester linkage of the sn-2 tail of phospholipids in the presence of calcium ions, producing lysophospholipids and fatty ac-

Figure 2. Porcine pancreas phospholipase A₂-induced release of carboxyfluorescein (CF) from unilamellar vesicles (filled symbols) and vesosomes (open symbols) for control (no PLA₂), 5 ng/mL PLA₂, and 10 ng/mL PLA₂ at 37 °C. The unilamellar vesicles were quickly degraded by the lipase, and CF was released in a dose-dependent fashion much faster than the control vesicles. Release from the vesosomes was not increased by the lipase, and there was no significant difference with release from the control vesosomes. Lines are a guide to the eye.

ids. This yields a rapid increase in membrane permeability and subsequent loss of the vesicle contents (Figure 2).³⁰ Unilamellar vesicles and vesosome samples were diluted with a calcium-containing buffer (85 mM NaCl, 50 mM TES, 10 mM CaCl₂, pH 7.4) to 0.1 mg/mL total lipid. PLA₂ (3 μg/mL stock solution) was added to the samples at the desired concentrations, and the same volume of buffer was added to the control samples. The initial fluorescence $I(0)$ was measured, and then each sample was divided into a series of 1 mL aliquots, which were then incubated at 37 °C in a water bath to induce PLA₂ enzymatic activity. At different times up to 20 h, aliquots were collected and their fluorescence was measured, producing the PLA₂-induced release profiles shown in Figure 2. The control vesicle release profile gives the permeability of the DPPC vesicles at 37 °C; the half-life for release is >20 h. PLA₂ increases the CF release in a concentration-dependent manner, reducing the half-life for release to ~3 h for 5 ng/mL PLA₂ and to ~1.5 h for 10 ng/mL PLA₂.

The multicompartment structure of the vesosome more than doubles the half-life for release without PLA₂. More importantly, PLA₂ does not significantly increase CF release from vesosome. After 20 h of exposure to PLA₂ at 37 °C, the vesosomes still retain the same amount of CF as the control vesosomes. This indicates that the PLA₂ cannot traverse the exterior bilayer of the vesosome to degrade the internal vesicles during the time of the experiment. While PLA₂ increases the permeability of the small-molecule CF (molecular mass of 376 Da) from unilamellar vesicles, apparently it takes much longer for PLA₂ (molecular mass of 14 kDa) itself to cross the exterior lipid bilayer of the vesosome.²⁴

Figure 3. Calf serum-induced release at 37 °C of carboxyfluorescein from unilamellar DPPC vesicles after mixing with various fractions of buffer. Serum induced rapid release from the vesicles compared to controls in a dose-dependent fashion. The release rate appeared to slow at longer times, suggesting that the enzymes or lipases responsible for the increased release rates were being consumed during the process. Lines are a guide to the eye.

While the exterior membrane of the vesosome is likely permeabilized to small molecules, it retains its ability to prevent the enzyme from interacting with the interior compartments. The compartment-within-a-compartment structure of the vesosome is sufficient to eliminate the effects of the lipase on CF release, even though the chemical composition of the interior or exterior bilayers of the vesosome is the same as that of the vesicles.

Serum is a more complex and biologically relevant medium, containing a variety of proteins, enzymes, and molecules that can interact with lipid membranes. Vesicles and vesosomes with 20 mM internal CF were diluted to 0.1 mg/mL lipid in different mixtures of newborn calf serum with buffer. The initial fluorescence, $I(0)$, and total fluorescence, I_{tot} were determined, and the remainder of each sample was divided into a series of 1 mL aliquots, which were incubated at 37 °C in a water bath. There was more variation between different experiments in serum than PLA₂; different batches of serum were more or less active in enhancing CF release. The data presented are representative experimental runs; in all cases, identical trends were seen between batches of serum.

Figure 3 shows the release profile from unilamellar vesicles in different serum/buffer mixtures. Serum induced faster release from the control vesicles in buffer; the release rate increased with increasing serum fraction. The total release from the vesicles at 10 h was proportional to the fraction of serum, suggesting that there was a limiting amount of whatever serum component that enhanced lipid degradation.¹⁶ Figure 4 shows that the corresponding release rates from vesosome samples were much slower than those from unilamellar vesicles. After 12 h, vesosomes released ~20% of the CF, independent of the serum/buffer ratio, while the unilamellar vesicles released 95% when exposed to 75% serum. Not only does the vesosome structure increase serum stability, but it also protects in a serum-

Figure 4. Calf serum-induced release at 37 °C of carboxyfluorescein from DPPC vesosomes after mixing with various fractions of buffer. Serum did not increase the release rate from the vesosomes within the time of this experiment compared to controls. The multicompartiment vesosome structure is responsible for the decrease in release, as the interior vesicles and exterior bilayer of the vesosome were made from DPPC, the same lipid that was used in the vesicles in Figure 3. Lines are a guide to the eye.

concentration-independent manner, indicating that the serum components have not been able to traverse the external membrane to reach the contents of the vesosome within 12 h.

In a second series of experiments, CF release into serum/buffer mixtures was followed for an extended period (Figure 5). The vesosome structure extended the half-life for release from vesosomes in serum to more than 50 h, and there appears to be a delay in release for

Figure 5. Calf serum-induced release at 37 °C of CF from DPPC vesosomes followed for 48 h. As in Figure 4, there is an initial delay of release, during which the exterior vesosome membrane effectively shields the internal compartments from degradation by serum. At later times, the rate of release increases but is still significantly slower than that from unilamellar vesicles (Figure 3). Over 70% of the CF is retained for 48 h in the vesosome. Lines are a guide to the eye.

MATERIALS AND METHODS

DPPC was purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Porcine pancreatic PLA₂ and Triton X-100 (RTX-100) were from Sigma (St. Louis, MO). Carboxyfluorescein, BODIPY 581/591 C₅-HPC, DHPE Biotin-X, and fluorescently labeled avidin (BODIPY FL) were from Invitrogen Molecular Probes (Carlsbad, CA). Bio-Mag particles labeled with streptavidin were from Qiagen (Valencia, CA). Size-exclusion chromatography was done with PD-10 columns (Amersham Bio-

~10 h before any significant amount of CF is released. There is some variability between serum samples in promoting release from the vesosomes; however, the differences in release between vesicles and vesosomes were significant for all serum samples examined. The serum components responsible for degrading the bilayers and increasing permeability were effectively shielded from the interior compartments for this initial lag period.

DISCUSSION

Previous work^{9,10,13–16,19} has shown that modifying the composition of unilamellar vesicles has little effect on contents release in physiological environments. However, adding a second barrier to the lipases and serum components responsible for premature release significantly increases the half-life of carboxyfluorescein in PLA₂ and in serum. This shows the benefits of the eukaryotic-mimic structure of the vesosome. The protection afforded the interior compartments by the exterior membrane is made obvious (1) by the inability of streptavidin or avidin to bind to the interior compartments, which we have used as a negative selection step in vesosome purification, and (2) by the absence of a permeability increase in the vesosome after exposure to phospholipase A₂ and serum. The additional bilayer membrane provides a physical barrier to direct interaction with the external medium and also efficiently delays CF leakage.

We expect that varying the internal vesicle membrane composition could further optimize contents release in serum and, perhaps, help identify the components of serum responsible for the membrane degradation that leads to premature drug release. For example, sphingomyelin-based internal capsules would be resistant to phospholipase degradation even after the lipase degraded the external barrier membrane. This encapsulation-protection concept could easily be applied to any colloidal delivery system that suffers from early degradation-induced drug leakage. The vesosome also offers the flexibility to deliver multiple drugs within a single carrier, which has been shown to offer important advantages in chemotherapy.²³ The vesosome structure also shows a potentially important advantage enjoyed by eukaryotes: the internal compartmentalization protects important organelles from interacting with a hostile environment.

sciences, Piscataway, NJ) pre-packed with an 8.3 mL bed volume of Sephadex G-25 between two sintered polyethylene frits of pore sizes 20–85 μm. DPPC vesicles (50 nm diameter) were prepared by standard extrusion methods using a Lipex Biomembranes Extruder (Vancouver, Canada).

Vesosomes were made by first inducing the transition from the normal (L_β) bilayer phase to the interdigitated (L_i) bilayer phase by dropwise addition of 0.106 mL of ethanol (3 M net ethanol concentration) to 0.5 mL of a 50 mg/mL DPPC vesicle sus-

pension, under stirring, at room temperature.³¹ The initially bluish vesicle suspension turned milky white, and its viscosity increased significantly. After 30 min of stirring, the interdigitated sheets were washed twice with an excess ethanol by adding 4 mL of buffer, followed by centrifugation and pellet collection. The interdigitated sheets are stable to the removal of the ethanol and replacement with buffer as long as $T < T_m$.^{29,31} The DPPC vesicles to be encapsulated were labeled with 0.02 mol % of the fluorescent dye b-BODIPY 581/591 C₅-HPC and 0.1 mol % of the specific receptor DHPE Biotin-X. Vesosomes with two different interior vesicle concentrations were prepared by mixing 0.5 mL of either 100 or 50 mg/mL of the labeled vesicles with the washed interdigitated sheets. The mixture was then heated at 46 °C—above the 41 °C main transition temperature of DPPC—for 20 min under gentle stirring, driving the sheets to close around the vesicles in suspension to form the vesosomes.

Freeze-fracture samples of the vesosomes were prepared by first depositing a film of sample liquid 100 μ m thick between two copper planchettes. The samples were frozen by plunging the sample 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 JEM Cryofract freeze-fracture device. After temperature (-170 °C) and pressure ($<10^{-7}$ Torr) equilibration, the sample was fractured, and the two resulting surfaces were replicated with 1.5 nm platinum deposited at a 45° angle, followed by 15 nm of carbon deposited normal to the surface. The copper planchettes were dissolved in a mixture of chromic acid, sulfuric acid, and water, and then the replicas were washed in water and allowed to stand in ethanol for several days to dissolve any remaining lipid. The replicas were collected on formvar-coated TEM grids (Ted Pella, Redding, CA).^{32–36} A Gatan CCD camera was used to record digital bright-field images using a FEI Technai transmission electron microscope at 100 keV.

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