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Undulating tubular liposomes through incorporation of a synthetic skin ceramide into phospholipid bilayers

Peng Xu^a, Grace Tan^a, Jia Zhou^a, Jibao He^b, Louise B. Lawson^c, Gary L. McPherson^d, and Vijay T. John^{a,*}

^a Department of Chemical & Biomolecular Engineering, Tulane University, New Orleans, LA 70118, USA

^b Coordinated Instrumentation Facility, Tulane University, New Orleans, LA 70118, USA

^c Department of Microbiology & Immunology, Tulane University School of Medicine, New Orleans, LA 70112, USA

^d Department of Chemistry, Tulane University, New Orleans, LA 70118, USA

Abstract

Non-spherical liposomes were prepared by doping *L*- α -phosphatidylcholine (PC) with ceramide VI (a skin lipid). Cryo-transmission electron microscopy shows the liposome shape changing from spherical to an undulating tubular morphology, when the amount of ceramide VI is increased. The formation of tubular liposomes is energetically favorable and is attributed to the association of ceramide VI with PC creating regions of lower curvature. Since ceramides are the major component of skin lipids in the stratum corneum, tubular liposomes containing ceramide may potentially serve as self-enhanced nanocarriers for transdermal delivery.

Introduction

Liposomes are colloidal entities in aqueous solution that consist of one or more lipid bilayers enclosing an inner aqueous phase. They are typically spherical with sizes ranging from 20 nm to 10 μ m.¹ The ability to encapsulate hydrophilic compounds such as proteins in the aqueous core of the liposomes and simultaneously incorporate lipophilic drugs in the hydrophobic lipid bilayer specifically renders liposomes as suitable vehicles for drug delivery.^{2, 3}

The majority of liposomes described in the literature are spherical in shape, e.g. those prepared from lipids such as phosphatidylcholine. Reports of non-spherical tubular liposomes with high aspect ratio are much less common, although helical liposomes have been reported as early as 1982 in mixtures of cardiolipin and phosphatidylcholine.⁴ Chiruvolu and coworkers reported on spontaneously formed spindle-shaped vesicles made of dimyristoylphosphatidylcholine, geraniol and water.⁵ Ishiyama and coworkers demonstrated that phosphoinositide forms helical tubular vesicles when stabilized by myelin basic protein in the presence of a nickel-chelating lipid.⁶ Anchoring of amphiphilic polymers is also reported to induce tubulation in lipid vesicles.^{7–9}

*Corresponding author. Department of Chemical & Biomolecular Engineering, Tulane University, New Orleans, LA 70118, USA. Tel: 1-504-865-5883, Fax: 1-504-865-6744. vijay.john@tulane.edu.

Supporting Information Available: Detailed experimental description of encapsulation of fluorescein sodium salt in tubular liposomes and extended figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Besides providing an alternative to traditional oral administration and invasive procedures such as needle injection, the presence of liposomes in a topical formulation has demonstrated the potential of improving penetration of actives.^{10–16} One primary example are flexible liposomes containing sodium cholate that deform and squeeze through the intercellular pathway of the stratum corneum, when driven by the osmotic gradient in the skin.¹⁷ In our present study, the synthetic skin lipid ceramide VI (Evonik Corp.) is incorporated as a dopant in phospholipid liposomes. Ceramides are the simplest sphingolipids, and are composed of either a saturated or mono-unsaturated fatty acid linked to a sphingosine base via an amide bond. They are some of the least polar and more hydrophobic lipids, thus providing the stratum corneum its barrier properties.¹⁸ Although ceramides account for approximately 50% lipid weight of the stratum corneum,¹⁹ they exist in much lower proportion in cell membranes, where they are intermediates of sphingosine metabolism and function in cell signaling.^{20, 21} Ceramide VI consists of a phytosphingosine backbone acylated with a long chain alpha-hydroxy stearic acid. It is a synthetic ceramide having the same stereochemical configuration as ceramides in human skin. Both the fatty acid and phytosphingosine base of ceramide VI are saturated, which differ from ceramides commonly observed in mammalian cell membranes. The structural similarity of ceramide VI to skin lipids may facilitate ceramide VI to participate in and perturb the intercellular lipid organization for the penetration of actives through the stratum corneum. In recent classifications of ceramides,²² ceramide VI has the same structure as the skin lipid ceramide 7.²³ The structure of the ceramide is shown in Scheme 1.

Ceramides have also generated immense interest due to their ability to trigger cell apoptosis in cancer therapy.^{24–28} Our motivation in the preparation of ceramide containing liposomes is to develop new vehicles for transcutaneous vaccine delivery. In the preparation of these liposomes, we have observed the systematic formation of tubular liposomes which we now report.

Results and Discussion

Ceramide VI containing liposomes were prepared using the lipid film hydration method. During the preparation procedure, ceramide VI (Evonik) and *L*- α -phosphatidylcholine from soybean (95%, Avanti Polar Lipids) were dissolved in a 2:1 (v/v) chloroform-methanol mixture. The dissolved lipid was completely dried on a rotary evaporator and the dried lipid film was hydrated with distilled water at 50 °C to obtain a 2% (w/v) liposome suspension. The liposome suspension was probe sonicated and subsequently extruded through a series of 400 nm and 100 nm pore size polycarbonate membranes at 55–65 °C to downsize the liposomes. Ceramides aggregating on the filter support of the extruder were discarded. The extruded liposome suspensions are translucent in appearance with a slight purple tinge in color. They remain stable for at least 2 months when stored at room temperature but can be alternatively stored at 4 °C to prolong their shelf-life. Cryo-transmission electron microscopy was utilized to image the liposomes in their native state. A 10 μ l drop of liposome suspension was placed on a holey carbon grid and rapidly vitrified in liquid ethane. The sample was then transferred under the protection of liquid nitrogen to the cryo-transmission electron microscope (cryo-TEM) sample holder and inserted into the cryo-TEM (JEOL 2011, Gatan). The temperature of the sample grids was maintained at –175 °C during the course of imaging.

The chemical structures of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, the predominant species in PC, and ceramide VI (cerVI) are shown in Scheme 1. The shape evolution from spherical to non-spherical and then to undulating tubular liposomes is best shown by increasing the ceramide VI content in a series of ceramide VI and PC containing liposomes (mol fraction of cerVI = mol cerVI/(mol PC + mol cerVI) = 0, 0.12, 0.35, 0.56, 0.75). From the captured image in Figure 1a, the control sample (PC liposomes containing no ceramide VI) consists almost entirely of spherical liposomes with diameters of 100 nm or less.

The few slightly oblong liposomes observed may be due to compression of the flexible bilayers in the thin liquid film prior to vitrification. Upon incorporation of ceramide VI (mol fraction of cerVI = 0.12), we observe an initial buckling of the liposome to a non-spherical morphology, as described in Figure 1b. There is a high possibility that ceramide VI is not homogeneously distributed between the liposomes in suspension, thus explaining the rich variety of liposome morphology present in the sample. Some of the effects of ceramides documented in the literature include inducing lipid ordering²⁹ and the formation of ceramide-rich domains in phospholipid bilayers,^{30–32} where segregation into these ceramide-rich and poor regions is dependent on the line tension and dipole-dipole repulsion between the lipids.³³ Due to the inhomogeneously distributed ceramide VI, liposomes containing minimal ceramide adopt an almost spherical shape (denoted by arrow heads) while liposomes with a larger amount of ceramide infiltrated into the lipid bilayers begin to elongate and form precursors for the undulating tubular structure (Figure 1b, black arrows). Ceramide VI has an inherently high phase transition temperature (>70°C).²³ Hence, the partitioning of ceramide VI into the liposome bilayer tends to rigidify the interface and cause an elongation in liposome morphology. The presence of undulating tubular liposomes becomes increasingly prominent as the mol fraction of ceramide VI reaches 0.35 (Figure 2a). During this stage, individual tubular liposomes fold over with their sinuous feature facilitating intertwinement to form 50 nm to 400 nm long liposomes. A higher magnification image of an intertwined undulating liposome is provided in Figure 2b. When the mol fraction of ceramide VI reaches 0.56, the presence of closed-end undulating tubules is the most prominent. This is notably the optimum ceramide VI content showing fully-developed tubular liposomes. Though some of the liposomes may be over 800 nm in length (Figure 2c), there is ultimately some variation between samples and liposome populations of 30–40 nm in width and 150 nm to 300 nm in length (Figure 3a) are also common. It is noticed that the liposomes are not necessarily intertwined, as observed in Figure 3a. Although the majority of liposomes remain tubular, ribbons (arrow) and short liposomes are occasionally present. Ceramide induced morphological alterations in membranes has been documented for palmitoylceramide where cylindrical structures with round extremities are present at a ceramide mol fraction above 0.5.³⁴ There appears to be an optimum threshold for the incorporation of ceramide VI as the yield of liposomes decreases beyond this point. When the ceramide VI content is increased to 75%, the liposome population sharply declines. Although the dominant morphology of the liposomes is still tubular, shrinkage in liposome diameter (Figure 3b) and the emergence of shorter undulating liposomes are observed. A plausible explanation for this is a shortage in PC since PC facilitates the incorporation of ceramide VI into the bilayers. Ceramides, due to their hydrophobicity and low polarity, cannot form aggregates in aqueous suspension.¹⁸

Phosphatidylcholine liposomes prepared by the lipid film hydration method are spherical. Therefore, the observed morphology changes suggest that ceramide VI plays an important role in the formation of tubular liposomes. The spontaneous radius of curvature of surfactant and lipid assemblies is expressed through the packing parameter, $\rho = v_t/a_h \cdot l_{c,t}$, where v_t is the volume of the hydrocarbon tail of the lipid; a_h is the area of the lipid head group; and $l_{c,t}$ is the critical chain length of the hydrocarbon tail.³⁵ Flexible bilayers or vesicles are formed when ρ ranges from 0.5 to 1, planar bilayers are obtained when $\rho \approx 1$ and reverse micelles are favored when $\rho > 1$. The ratio $v_t/l_{c,t}$ approaches 0.21 nm² for single tail surfactants and 0.42 nm² for double tail lipids.³⁵ The packing parameter of PC is 0.7 (a_h of PC is approximately 60 Å²),^{36, 37} consistent with our observation that PC forms spherical liposomes. Ceramide VI on the contrary, has a packing parameter of 1.2 (a_h of cer6II is approximately 35 Å²). The high packing parameter of ceramide VI implies that these compounds may form structures with curvatures around water (such as in reverse micelles in an organic solvent). In aqueous systems, the high packing parameters indicate that ceramides would cause a flattening of the bilayer curvature or perhaps even the opposite curvature to normal phospholipid vesicles. Additionally, there is a non-uniform distribution of ceramide in the ceramide VI-containing liposomes with

domains rich in ceramide and domains poor in ceramide (reflecting the curvature of just PC liposomes). Multilamellar vesicles consisting of palmitoylceramide and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine have also been documented to contain ceramide-rich and ceramide-poor domains where a long range effect is observed by influencing properties of phosphocholine-rich regions.³⁴ The packing parameter of the different domains would differ (due to the different proportion of PC and ceramide in these domains), thus inducing dissimilarities in the local bilayercurvature, giving rise to the tubular wavy structure of ceramide VI-containing liposomes. Since the flatter bilayer structure is promoted by ceramides, it is proposed that ceramide VI is enriched in the cylindrical surface while the spherical endcaps of the liposomes are enriched in PC. The sensitivity of bilayer structural morphology to differences in headgroups, such as those of galactosylceramides is well-documented in the literature.^{38, 39} Similarly, the disparity between head groups of ceramide VI and PC, as well as possible intermolecular hydrogen bonding between ceramide VI - ceramide VI molecules in ceramide-rich domains and ceramide VI - PC molecules in the lipid bilayer is expected to influence the local bilayer curvature.

When the liposome's bending free energy is considered, it is energetically favorable to adopt a tubular conformation if the following criteria are satisfied: the ratio of l/R should be greater than 5.56, where l and R are the length and radius of the tubular liposomes.⁴⁰ The diameters and lengths of the tubular liposomes are measured as 30–40 nm and 150–300 nm respectively (Figure 3c), resulting in $l/R > 7.5$, which suggests that the helical liposomes are stable. Static and dynamic light scattering analysis shows that the radius of gyration divided by the hydrodynamic radius (R_g/R_h) increases from 0.61–0.67 for spherical PC liposomes to 0.88–1.06 for 0.5 weight fraction ceramide VI liposomes. The rise in value of R_g/R_h suggests that a change in morphology from spheres to elongated structures has occurred. Therefore, the unique morphology of the ceramide VI doped liposomes may be attributed to the differences in bending free energy, packing parameters, and polar interactions among the lipid head groups.

Although there is a certain variation in sample quality amongst different batches of ceramide VI obtained from the supplier, the trend to form undulating tubular liposomes with increasing ceramide VI incorporation holds true, with a more prominent yield of tubular liposomes when the hydration and extrusion temperature is increased to 80 °C. A tendency for the liposomes to straighten and become less undulated at the higher temperatures is also observed. This is because ceramide VI has a relatively high phase transition temperature,²³ and a larger proportion of ceramide VI is incorporated into the liposomes when a higher temperature is used, thus promoting the flatter curvatures. Experiments where the amount of PC is held constant while increasing ceramide VI up to the optimum content were also performed and shows a similar trend of increased tubular liposomes. Although the liposomes described earlier were prepared in distilled water, tubular liposomes have also been prepared in 1X phosphate buffered saline.

Scheme 2 illustrates a summary of the shape evolution of liposomes as the ceramide content is increased. Although the actual process may not be as simplistic, the above is a feasible route detailing changes in morphology. We envision these undulating tubular liposomes to have potential applications in transdermal delivery where they may serve as drug nanocarriers by penetrating through the stratum corneum when combined with skin hydration.

Alternatively, non-spherically shaped liposomes have a longer physiological lifetime compared to their spherical counterparts⁴¹ and may have different drug release profiles from those of conventional spherical liposomes. Preliminary experiments to demonstrate that tubular liposomes can entrap a water soluble marker (fluorescein sodium salt) were performed. The marker was dissolved in 1X PBS and used to hydrate the dried lipid film. Upon ultracentrifugation of the tubular liposome suspension to separate the unencapsulated marker

from the liposomes, the liposomes pelletized as an orange mass signifying entrapment of the marker in the liposomes. Experimental details and optical micrographs can be found in the supporting information. In continuing work, we will also report the ceramization of such tubular liposomes through the formation of silicas using the liposome as templates for such nanostructures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Almost all phosphatidylcholine liposomes are spherical. (mol fraction of ceramide VI = mol ceramide VI/(mol PC + mol ceramide VI) = 0). (b) Upon addition of ceramide VI, buckling of the liposomes to an aspherical shape is observed. (mol fraction of ceramide VI = 0.12). Liposomes containing minimal ceramide adopt an almost spherical shape (arrow heads) while those rich in ceramide pucker up (double arrows) or elongate to form precursors for the undulating tubular structure (single arrow).

Figure 2.

(a) With progressive addition of ceramide VI, transition to elongated undulating liposomes is observed (mol fraction ceramide VI = 0.35). (b) Higher magnification image of an intertwined undulating liposome (mol fraction ceramide VI = 0.35). (c) The optimum ceramide VI content is reached at 0.56 mol fraction ceramide VI where well-defined elongated liposomes are abundant in the sample.

Figure 3.

(a) An alternate sample for 0.56 mol fraction ceramide VI. Elongated liposomes 30–40 nm wide and 150 nm to 300 nm long are typically observed. A few shorter liposomes and ribbons (arrow) are also present. (b) Shrinkage in liposome population and liposome dimension occurred when the mol fraction of ceramide VI is increased to 0.75.

Scheme 1.

Chemical structures of (A) 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine and (B) ceramide VI.

Scheme 2.

Shape evolution of ceramide VI and PC liposomes as ceramide VI content is gradually increased from a ceramide VI mol fraction of 0 to 0.75.