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Formation of homogeneous unilamellar liposomes from an interdigitated matrix

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Abstract

Phospholipid–ethanol–aqueous mixtures containing bilayer-forming lipids and 20–50 wt.% of water form viscous gels. Further hydration of these gels results in the formation of liposomes whose morphology depends upon the lipid type. Upon hydration of gels containing mixtures of the lipids 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), small homogeneous and unilamellar liposomes were produced. In contrast, hydration of gels containing only POPC resulted in formation of large multilamellar liposomes. Likewise, mulitlamellar liposomes resulted when this method was applied to form highly fusogenic liposomes comprised of the novel negatively charged *N*-acyl-phosphatidylethanolamine (NAPE) mixed with di-oleoyl-phosphatidylcholine (DOPC) (7:3) [T. Shangguan, C.C. Pak, S. Ali, A.S. Janoff, P. Meers, Cation-dependent fusogenicity of an *N*-acyl phosphatidylethanolamine, Biochim. Biophys. Acta 1368 (1998) 171–183]. In all cases, the measured aqueous entrapment efficiencies were relatively high. To better understand how the molecular organization of these various gels affects liposome morphology, we examined samples by freeze-fracture transmission electron microscopy and X-ray diffraction. We found that phospholipid–ethanol–water gels are comprised of highly organized stacks of lamellae. A distinct feature of the gel samples that result in small unilamellar liposomes is the combination of acyl chain interdigitation and net electrostatic charge. We speculate that the mechanism of unilamellar liposome formation proceeds via formation of stalk contacts between neighboring layers similar to membrane hemifusion intermediates, and the high aqueous entrapment efficiencies make this liposome formation process attractive for use in drug delivery applications.

Keywords: Homogeneous; Liposome; Matrix

1. Introduction

Self-assembly of amphiphiles into aggregates of different morphologies has been of interest across biological, chemical and physical sciences for several decades. The ability to control self-assembly in order to produce nanoparticles of desired morphology and properties is vital for development of future nanotechnology and drug delivery. In particular, liposomes have been widely explored as drug delivery vehicles due in part to the ease with which their assembly can be manipulated to produce specifically designed carriers for a variety of different applications. Additionally, liposomes are also appealing because of their biocompatibility, ability to deliver either aqueous or hydro-

Abbreviations: POPC, palmitoyl-oleoyl-phosphatidylcholine; POPG, palmitoyl-oleoyl-phosphatidylglycerol; DOPC, di-oleoyl-phosphatidylcholine; NBD-PE, nitrobenzoxadiazol-phoshatydylethanolamine; NAPE, *N*-acyl-phosphatidylethanolamine; DPPC, di-palmitoyl-phoshatidylcholine; DSPC, di-stearoyl-phosphatidylcholine; SUV, small unilamellar vesicles

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phobic therapeutics, including small molecules and large biomolecules such as DNA, and their ability to accommodate various ligands/coatings on their surface.

For the efficient entrapment of aqueous drug in liposomes, a variety of liposome formation methods have evolved; for a review, see Lasic and Papahadjopoulos [2]. In order to be encapsulated, most aqueous drugs must be included in the buffer solution during vesicle formation. For use as carriers in the systemic circulation, it has been established that liposome size should be limited to less than 200-nm diameter in order to maximize circulation lifetime [3]. Therefore, it is most desirable to form liposomes near 200 nm in diameter to avoid further processing (e.g., size reduction by extrusion [4]) which inevitably adversely affects retention of encapsulated water-soluble compounds. Although there are a few examples of 'active' or 'remote' loading processes which are highly efficient (>95%), these techniques, which involve loading of pre-formed liposomes, are limited to ionizable membrane-permeable molecules such as cationic anthracyclines [5,6].

We describe here a process by which liposomes can be produced, which exhibit both a high encapsulation efficiency and a desirable size distribution. This process involves the stepwise hydration of lipid dissolved at high concentrations in ethanol [7,8]. Recently, several other laboratories also reported the efficient encapsulation of DNA and polynulceotides (70–80%) in liposomes less than 200 nm in diameter using relatively high ratios of ethanol to lipid/water [9–11]. Lipid compositions and protocol details varied suggesting that the common link, ethanol, plays a critical role in these vesicle formation processes. While no mention of the pre-liposome characteristics was made in the above cited reports, we found that our lipid–ethanol–aqueous mixtures formed a viscous gel prior to liposome formation.

A similar viscous gel was previously observed when we added ethanol to small unilamellar vesicles (SUVs) comprised of saturated chain lipids (e.g., DPPC, DSPC) [12–14]. Evaluation of those particular intermediary gels revealed that the precursor SUVs had fused into stacked planar interdigitated-bilayer sheets which formed large liposomes upon heating the suspension to above the gel-to-liquid crystalline phase transition temperature of the lipid. We established that without the induction of interdigitation by ethanol, or by hydrostatic pressure [15], those gels would not form. While in the current study we are using unsaturated lipids which are not expected to undergo interdigitation [16], there has been evidence that monounsaturated lipids are capable of ethanol-induced acyl chain interdigitation under the appropriate conditions [17]. To gain more insight into the nature of the lipid–ethanol–aqueous gels and their influence upon the resulting liposome morphology, we detailed the conditions for gel and liposome formation and explored the molecular organization and structural transitions of phospholipid–ethanol–water mixtures by freeze-fracture electron microscopy and X-ray diffraction.

2. Materials and methods

POPC, POPG, DOPC, *N*-dodecanoyl-di-oleoyl-phosphatidylethanolamine (referred to here as NAPE) and nitrobenzoxadiazol-phoshatidylethanolamine (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL); cholesterol, all buffer salts and solvents were obtained from Sigma-Aldrich (St. Louis, MO). All buffer salts were Sigma Ultra grade. Deionized water and HPLC grade solvents were used in all experiments.

To prepare lipid–ethanol–buffer mixtures, dry powdered lipids were dissolved in appropriate amounts of ethanol. For samples subjected to lamellarity analysis, the fluorescent lipid NBD-PE was included at 0.2 mol% of total lipid. After the lipids were completely dissolved in ethanol, appropriate amounts of buffer were added and the mixtures vigorously vortexed.

2.1. Compositional analysis of lipid–ethanol–water gels

In samples exhibiting macroscopic phase separation, the fluid and gel portions were analyzed for lipid and ethanol as a function of temperature. Samples were placed in microcentrifuge tubes and incubated in a circulating water bath at each given temperature for 30 min. Samples were then centrifuged for 15 min at 2000 rpm in a temperature-controlled Eppendorf microcentrifuge, equilibrated at appropriate temperature, and placed in a water bath for an additional 15-min incubation. About 10-μL aliquots of fluid and gel portions of the samples were taken, placed into snap-closure microcentrifuge tubes and immediately sealed. After the weight of the aliquots was recorded, 1 ml of deionized water was placed into each tube, the contents were mixed and the tubes were kept tightly sealed. Phosphatidylcholine concentration in the samples was determined by choline assay (Wako Diagnostics, Richmond, VA). Ethanol concentration was determined using an ethanol kit assay (Sigma-Aldrich). Amount of water in each aliquot was deduced from the weight balance of all components.

2.2. Liposome analysis

Size of the liposomes was measured by dynamic light scattering using Nicomp Submicron Particle Sizer 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA). Lamellarity of liposomes was measured using a NBD-PE dithionite reduction assay [18] in a microplate format. Briefly, the liposomes were placed in microplate wells at approximately 1 mM lipid concentration. Each sample was pipetted into six wells, of which three wells were treated with dithionite and three wells served as controls. Dithionite solution was pipetted into the appropriate wells of the microplate, contents were quickly mixed and the measurements were initiated immediately. Fluorescence intensity was monitored over a period of at least 30 min,