Liposome Methods and Protocols

METHODS IN MOLECULAR BIOLOGY™

Liposome Methods and Protocols

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Preface

In vitro utilization of liposomes is now recognized as a powerful tool in many bioscience investigations and their associated clinical studies, e.g., liposomes in drug targeting; liposomes in gene transport across plasma and nuclear membranes; liposomes in enzyme therapy in patients with genetic disorders. However, before these areas can be effectively explored, many basic areas in liposome research require elucidation, including: (a) attachment of liposomes to cell surfaces; (b) permeation of liposomes through the plasma membranes; and (c) stability of liposomes in cell or nuclear matrices. None of these areas have been exhaustively explored and liposome researchers have ample opportunities to contribute to our knowledge.

The aim of *Liposome Methods and Protocols* is to bring together a wide range of detailed laboratory protocols covering different aspects of liposome biology in order to assist researchers in those rapidly advancing medical fields mentioned earlier. With this goal in mind, in each protocol chapter we have detailed the materials to be used, followed by a step-by-step protocol. The Notes section of each protocol is also certain to prove particularly useful, since the authors include troubleshooting tips straight from their benchtops, valuable information that is seldom given in restricted methods sections of standard research journals. For this reason we feel that the book will prove especially useful for all researchers in the liposome field.

In editing *Liposome Methods and Protocols*, we attempted to cover as many biochemical areas as the technique addresses, as the Contents demonstrates. We should mention here only that the reader will find a good cross-section of the commonly used liposome techniques, as well as certain more sophisticated techniques and protocols. Many readers will also find the current reference lists at the end of each chapter as a valuable source of background information.

We would like to thank all the authors for their fine contributions. The wide range of protocols they have so superbly realized will ensure that this is an indispensable book for researchers across many fields, including glycoproteins, glycolipids, glycosyltransferases, drug transport, viral transport, antibody delivery, synthetic peptide delivery to cells, and protease delivery.

Putting our personal convictions aside, however, we must leave final judgment of the book to the proper scientific communities, and we do so with confidence. We gratefully acknowledge the tireless help of Mrs. Dorisanne Nielsen during our editing of this book. We extend our thanks and appreciation to Dr. Asoke Shukla for his initial inspiration to edit this book.

Subhash Basu
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I

Physical Properties

1

Preparation, Isolation, and Characterization of Liposomes Containing Natural and Synthetic Lipids

Subroto Chatterjee and Dipak K. Banerjee

1. Introduction

The specificity, homogeneity, and availability of large-batch production of liposomes with natural lipids and synthetic lipids have made them an extremely useful tool for the study of diverse cellular phenomena, as well as in medical applications. In many cases, however, the success of the use of liposomes as drug carriers or vaccines and in gene delivery depends entirely on both their formulation and the method of preparation.

Liposomes are synthetic analogues of natural membranes. Consequently, in view of the fact that the lipid composition of the cell membrane is fixed, the general concept in the preparation of liposomes is to modify combinations of these lipid mixtures (to emulate the natural membrane) in the presence or absence of a variety of bioactive molecules with diverse functions. The methods for the preparation, isolation, and characterization of liposomes are as diverse as the applications of these molecules in health and disease. Accordingly, we feel it is a daunting task to cover each and every method that has been described for preparing liposomes. Thus, in this chapter we have focused on the preparation of three classes of liposomes, namely, the multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), and large unilamellar vesicles (LUVs). Several excellent books on liposome technology and its application in health and disease *(1–3)* have been published over the last decade. Readers are suggested to consult these works to obtain more information on an individual method relevant to the needs of their studies.

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2. Materials

1. Cholesterol is commercially available from several sources, for example, Avanti Polar Lipids (Alabaster, AL), Matreya, Inc. (Pleasant Gap, PA).
2. Dicetylphosphate (DCP) is available from KMK Laboratories (Fairview, NJ), Sigma Chemical Co. (St. Louis, MO), and Pierce Biochemicals, (Milwaukee, WI).
3. Dimyristoyl phosphatidylcholine (DMPC) is available from Avanti Polar Lipids, Sigma Chemical Co., CalBiochem-Behring (San Diego, CA), Pierce Biochemicals, and Matreya, Inc. Several other phospholipids and glycosphingolipids are available commercially in high quality from Matreya, Inc. as well. (*see* **Note 1**).
4. Organic solvents, typically chloroform (JT Baker, Phillipsburg, NJ), are used in the solubilization of a variety of lipids. However, often a small amount of methanol is also required to solubilize gangliosides and relatively polar lipids, such as phospholipids. Both chloroform and methanol are available commercially. Because chloroform can deteriorate on storage for more than 1–3 mo, it is a routine practice in many laboratories to redistill chloroform before use in a variety of biochemical experiments but in particular in liposome preparation. Subsequent to distillation, 0.7% ethanol is added as a preservative. Pear-shaped boiling flasks manufactured by Lurex Scientific Inc. (Vineland, NJ) have been recommended by some investigators for use because they have the best shapes for the distillation of organic solvents ***(4)***. Microbeads used for the distillation of solvents are commercially available from Cataphote Division of Ferro Corp. (Cleveland, OH and Jackson, MS).

3. Methods

3.1. Preparation of Multilamellar Liposomes

The strategy for preparation of MLVs is to use well characterized lipids in order to produce well defined liposomes ***(4)***. Equally important is the selection of bilayer components for toxicity and for shelf life optimization. The lipids normally used are the unsaturated egg phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), and the saturated lipids DMPC, dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidic acid (DPPA), and dipalmitoyl phosphatidylglycerol (DMPG). Stearylamine is used when cationic liposomes are preferred; and natural acidic lipids, such as phosphatidylserine (PS), PG, phosphatidylinositol (PI), PA, and cardiolipin (CL) are added when anionic liposomes are desired, while cholesterol is often included to stabilize the bilayer. Small amounts of antioxidants such as α-tocopherol or β-hydroxytoluidine (BHT) are included when polyunsaturated neutral lipids are used. A general protocol to prepare MLV is as follows:

1. Prepare a suitable solution of the lipid component in a pear-shaped flask (lipid concentrations between 5 and 50 m*M* in either chloroform or in chloroform–

methanol (3 : 1, v/v), and filter the mixture to remove minor insoluble components or ultrafilter to reduce or eliminate pyrogens.

2. Employing a rotary evaporator, remove the solvent, while maintaining a temperature of ~40°C in a water bath under negative pressure (*see* **Note 2**). Other methods of drying include spray drying and lyophilization *(5)*. Traces of organic solvents are removed employing a vacuum pump, normally overnight at pressures below milliTorr (~0.1 Pa). Alternatively, the sample may be dried under a very low vacuum (<50 μmol/mg) for 1–2 h in a dessicator with drierite™ (Fisher Scientific, Malvern, PA).
3. Subsequent to drying, 100 μL of 0.5 mm glass beads are added to the 10-mL flask containing the dried lipid mixture, and hydration fluid (0.308 *M* glucose), which is equal to the final volume of the liposome suspension, is added. Typically, the volume of hydration fluid used is determined by the amount of liposomal phospholipid and is usually in millimolars with respect to the hydration fluid *(1)*.
4. Vortex mixing the flask for 1–2 min causes all of the dried lipid from the flask to be dispensed into the hydration fluid. Alternative hydration mediums are distilled water, buffer solution, saline, or nonelectrolytes such as a sugar solution. For an in vivo preparation, physiological osmolality (290 mosmol/kg) is recommended and can be achieved using 0.6% saline, 5% dextrose, or 10% sucrose solution. MLVs of tens of micrometers to several tenths of a micrometer are spontaneously formed when an excess volume of aqueous buffer is added to the dry lipid and the flask is agitated.
5. The "dry" lipid mixture is then hydrated in an aqueous medium containing buffers, salts, chelating agents, and the drug to be entrapped (*see* **Note 3**).

3.2. Preparation of Small Unilamellar Liposomes

High-energy sonic fragmentation processes were introduced in the early 1960s *(6)* Refinements of these procedures using a high-pressure homogenization device followed *(7,8)*. SUVs are prepared by the following methodology to disperse phospholipids in water to form optically clear suspensions.

3.2.1. Sonication

Methods for the preparation of sonicated SUVs have been reviewed in detail by Bangham and others *(8)*. Typically the MLV dispersion is placed in test tubes and sonicated either in a bath sonicator or by tip sonication. Normally a 5–10-min sonication procedure (above T_c) is sufficient to prepare SUVs with radii < 50 nm. With some lipids, radii < 20 nm are also possible while some diacyl cationic lipids (including 1-[2-(oleoyloxy)-ethyl-2-oleoyl-3-(2-hydroxyethyl) imidarolinium chloride (DOIC) and dioctadecylamidoglycylspermine (DOGS) can even form micelles. Dioctadecyl diammonium bromide (DOBAD) neutral lipid liposomes cannot be sized <130 nm (*see* **Note 4**).