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
Manju Basu

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Preparation and Use of Liposomes for the Study of Sphingolipid Segregation in Membrane Model Systems

Massimo Masserini, Paola Palestini, Marina Pitto, Vanna Chigorno, and Sandro Sonnino

1. Introduction

Several investigations, carried out in either artificial or cellular models and using a variety of techniques ***(1–3)***, confirmed the prediction of Singer and Nicholson ***(4)*** about the presence of domains in biological membranes, that is, of zones where the concentration of the components and the physicochemical properties differ from the surrounding environment. Some domains have been better characterized in terms of the morphological, compositional, and functional aspects. This is the case for caveolae, flask-shaped invaginations of the plasma membrane, characteristically enriched in proteins of the caveolin family ***(5)***. However, the techniques used to isolate caveolae, when applied to cells apparently lacking caveolin, lead to the isolation of membrane fractions (caveolae-like) having characteristics in common with caveolae, such as their peculiar protein and lipid composition ***(6–9)***. In fact, caveolae and caveolae-like domains are enriched with functionally related proteins, suggesting a role of these domains in the mechanisms of signal transduction, cell adhesion, and lipid/protein sorting ***(6)***. Among lipids, sphingolipids (namely glycolipids and sphingomyelin) and cholesterol are characteristically enriched. In particular, GM1 ganglioside ***(10)*** has been proposed as a marker for these membrane structures in cells where this glycolipid is expressed. The peculiar lipid composition has suggested the involvement of glycolipid-enriched domains ("rafts") in lipid/protein sorting at the trans-Golgi network (TGN) level, and, in general, in all cell membranes ***(11)***.

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Preparation of model membranes mimicking the lipid assembly of caveolae and caveolae-like domains is available and is fundamental in order to study the biochemical, functional, and architectural features of domains. In recent years, several investigations clarified the fundamental features of sphingolipid domain formation in model membranes.

In this chapter, preparation of phospholipid vesicles containing sphingolipids in different segregation states is described. For this purpose, some known features affecting their segregation properties are taken into account. First, it is known that glycolipid segregation increases with increasing number of saccharide units ***(1,12,13)***. In this respect, GD1a ganglioside has a strong tendency toward lateral phase separation; and, for this reason, preparation of monolamellar phospholipid vesicles containing GD1a domains is described. Second, the segregation of sphingolipids depends on their ceramide moiety: when ceramide length and unsaturation are different from the membrane environment, glycolipids undergo domain formation. This has been demonstrated in model membranes ***(14)*** and in rabbit brain microsomal membranes ***(14,15)***. For this reason, and given the central role of GM1 ganglioside in caveolae and caveolae-like domains, the preparation of monolamellar phospholipid vesicles containing GM1 ganglioside domains is described. Third, the formation of sphingolipid domains depends on the presence of cholesterol. This occurrence has been reported for a large number of cellular systems ***(16)*** and in model membranes ***(17)***. For this reason, the preparation of monolamellar phospholipid vesicles containing glycolipids, cholesterol, and sphingomyelin domains is described. Starting from these experimental premises, this chapter describes the preparation of monolamellar liposomes of 100 nm diameter, in which different types of domains are realized, simply varying the nature and the proportion among the components.

In brief, after mixing lipids in organic solvent in the preestablished proportions, the solvent is evaporated and a lipid film is formed on the walls of a test tube. Lipids are soaked in buffer at a temperature higher than the gel to liquid-crystalline temperature transition of the lipid mixture. Finally, lipid mixtures are extruded 10 times, always at a temperature above the gel to liquid-crystalline temperature transition, through two stacked filters having controlled pores of 100 nm.

2. Materials

1. Thin-layer chromatography (TLC) plates, RP-8 high-performance liquid chromatography (HPLC) columns, and silica gel 100 for column chromatoghraphy are available from Merck GmbH. Filters (100 nm pore size) can be purchased from Nucleopore (Pleasanton, CA, USA).
2. Deionized water was distilled in a glass apparatus.

3. Phospholipids and cholesterol: Dipalmitoylphosphatidylcholine (DPPC), palmitoylsphingomyelin (SM), and cholesterol are available from Avanti Polar Lipids. All lipids can be stored at –20°C, either in a dried state or in stock solutions in chloroform–methanol (2:1 v/v), and are stable for several months at –20°C under nitrogen.
4. Gangliosides: Gangliosides GM1 and GD1a can be either prepared by fractionation of the total ganglioside mixture extracted from mammal brains by the tetrahydrofuran–phosphate buffer and purified from the glycerolipid contamination by partitioning with diethyl ether ***(18)*** followed by an alkaline treatment ***(19)***, or purchased from suppliers. Ganglioside molecular species of GM1 and GD1a with homogeneous ceramide moieties can be prepared by reversed-phase HPLC. The purity of gangliosides is very important. Spend some time to check for their purity: small impurities can have a large impact on the final result. Purity can be easily checked by TLC. Gangliosides must be stored at –20°C as dried powder.
5. 0.05 *M* Sodium acetate, 1 m*M* $CaCl_2$, pH 5.5.
6. *Clostridium perfringens* sialidase.
7. LiChroprep RP18 column.
8. *p*-Dimethylaminobenzaldehyde.
9. 10% Ammonium sulfate.

3. Methods

3.1. Lipids

3.1.1. Assay and Assessment of Purity of Phospholipids

The assay of phospholipid amount can be carried out spectrophotometrically by assaying the phosphorus content ***(20)***. The purity of phospholipids is very important. Purity can be easily checked by TLC. For this purpose, the TLC plate is overloaded with approx 15 nmol of a single lipid. The plate is developed with chloroform–methanol–water (60:35:4, by vol), and stopped when the solvent is at 0.5 cm from the top of the plate, usually after 20 min. Visualization of the phospholipid is carried out with a spray reagent to detect phosphorus ***(21)***. Only one spot must be visible in the TLC under these conditions.

3.1.2. Preparation of Ganglioside GM1

Ganglioside GM1 is 10–20% (molar) of the total ganglioside mixture from most mammalian brains. The GM1 content can be increased by treatment of the ganglioside mixture with bacterial sialidase. This treatment acting on the ganglioside sialosyl chains transforms the polysialogangliosides into GM1 ***(22)***.

1. The ganglioside mixture is dissolved (40 mg/mL) in prewarmed (36°C) 0.05 *M* sodium acetate, 1 m*M* $CaCl_2$ buffer, pH 5.5.
2. *Clostridium perfringens* sialidase (50 mU/g of ganglioside mixture) is added to the solution every 12 h. Incubation at 36°C is maintained for 2 d while stirring.

3. The sialidase-treated ganglioside mixture is then applied to a LiChroprep RP18 column (3–4 mL gel/g of ganglioside mixture) and, after washing with water to remove salts and free sialic acid, the gangliosides are eluted with methanol.
4. The methanolic solution is dried, dissolved in the minimum volume of chloroform–methanol–water (60 : 35 : 8 by vol), and applied to a silica gel 100 column (180–200 mL of gel/g of ganglioside mixture) chromatography, equilibrated, and eluted with the same solvent system; the chromatography elution profile is monitored by TLC (*see* **Subheading 3.1.5.**).
5. Fractions containing GM1 are collected, dried, and the residue dissolved in the minimum volume of propan-1-ol–water (7 : 3 v/v), and precipitated by adding four volumes of cold acetone.
6. After centrifugation (15,000*g*) the pellet is separated from the acetone and dried under high vacuum. By this procedure GM1 is obtained with homogeneity > 99.9% (assessed by TLC; *see* **Subheading 3.1.5.**). This procedure is suitable for a very large range of ganglioside mixture amounts, from a few milligrams to several grams.

3.1.3. Preparation of Ganglioside GD1a

GD1a is the main ganglioside of the ganglioside mixtures from mammalian brains, covering 30–45% as molar fraction of the total ganglioside mixture.

1. The ganglioside mixture is dissolved in the minimum volume of chloroform–methanol–water (60 : 35 : 8 by vol) and applied to a silica gel 100 column chromatography (300–320 mL of gel/g of ganglioside mixture), equilibrated, and eluted with the same solvent system; the chromatography elution profile is monitored by TLC (*see* **Subheading 3.1.5.**).
2. Fractions containing GD1a are collected, dried, and the residue subjected to a further chromatographic purification using the same conditions described in the preceding.
3. Fractions containing only GD1a are collected, dried, and the residue dissolved in the minimum volume of propan-1-ol : water (7 : 3 v/v), and precipitated by adding four volumes of cold acetone.
4. After centrifugation (15,000*g*) the pellet is separated from the acetone and dried under high vacuum. By this procedure GD1a with homogeneity > 99.9% is prepared (by TLC analysis; *see* **Subheading 3.1.5.**). This procedure is suitable to be adapted to a very large range of ganglioside mixture amounts.

3.1.4. Preparation of GM1 and GD1a Ganglioside Species Homogeneous in the Lipid Portions

Gangliosides GM1 and GD1a purified from brain gangliosides are characterized by a high content of stearic acid (> 90% of the total fatty acid content) and by the presence of both the molecular species containing C_{18}- and C_{20}-sphingosine (94–96% of the total species). Thus, by reversed-phase HPLC,

each ganglioside homogeneous in the oligosaccharide chain is fractionated mainly into two species containing stearic acid and C_{18}- or C_{20}-sphingosine ***(18,23)***. Reversed-phase chromatographic columns show very high resolution in separating the ganglioside species differing in the length of sphingosine, only when a small amount of ganglioside is loaded. We suggest to load a 25×4 cm column with a quantity of 5–6 μmol of ganglioside.

1. Five-micromole portions of GM1 or GD1a are dissolved in 1 mL of acetonitrile–water (1 : 1 v/v), and applied to a reversed-phase LiChrosphere RP8 column, 25×4 cm internal diameter, 5 μm average particle diameter (Merck, Darmstadt, FRG) through a syringe-loading sample injector equipped with a 1-mL loop.
2. Chromatography is carried out at 20°C with the solvent mixtures: acetonitrile–5 m*M* phosphate buffer, pH 7.0, in the ratio of 3 : 2 and 1 : 1 for GM1 and GD1a, respectively. The flow rate is 13 mL/min and the elution profile is monitored by flow-through detection of UV absorbance at 195 nm. The overall procedure requires about 90 min.

3.1.5. Ganglioside Homogeneity

1. Twenty to thirty micrograms of GM1 or GD1a, heterogeneous in the ceramide moiety, are applied for a width of 3–4 mm on silica gel HPTLC plates, then developed with the solvent system chloroform–methanol–0.2% aqueous $CaCl_2$ (50 : 42 : 11 by vol).
2. Twenty to thirty micrograms of GM1 or GD1a species, homogeneous in the ceramide moiety and containing C_{18}- or C_{20}-sphingosine, are applied as a 3–4 mm line on reversed-phase RP18-HPTLC plates, then developed 2 times with the solvent system methanol–acetonitrile–water (18 : 6 : 1 by vol).
3. After TLC, the gangliosides are made visible by treatment with an anisaldehyde reagent followed by heating at 140°C for 15 min ***(24)***, with a *p*-dimethylamino-benzaldehyde reagent followed by heating at 120°C for 20 min ***(25)***, and with 10% ammonium sulfate followed by heating up to 160°C. Quantification of the ganglioside spots is performed with a densitometer.

3.1.6. Ganglioside Assay

Ganglioside concentrations can be assessed using the sialic acid Svennerholm's assay ***(26)***.

3.1.7. Preparation of Stock Solutions of Lipids

Separate stock solutions are prepared in chloroform–methanol (2 : 1 v/v) containing 100 μmol/mL of one of the following lipids: DPPC, SM, or cholesterol. Prepare stock solutions of gangliosides containing 10 μmol/mL in chloroform–methanol (2 : 1 v/v).

3.2. Liposomes

3.2.1. Liposomes Composed of DPPC, Containing GD1a Ganglioside Domains

The main characteristics of these liposomes are the following: size 100 nm (1000 Å); shape monolamellar; gel to liquid-crystalline temperature transition (T_m) 42.5°C, determined by high-sensitivity differential scanning calorimetry. Therefore, the physical state up to this temperature is gel, and this feature should be taken into account anytime the physical state is important for the particular experiment to be performed.

For the preparation of these liposomes, containing 10% molar ganglioside, mix 90 µL of the stock solution of DPPC with 100 µL of the stock solution of GD1a ganglioside and proceed as described in **Subheading 3.3.** The approximate final concentration of liposomes is 9 µmol of phospholipid/mL, 1 µmol of ganglioside/mL. The exact final concentration should be checked by phospholipid and sialic acid assay. The reference temperature for this mixture, important for the preparation of liposomes, is 45°C.

3.2.2. Liposomes of DPPC, Containing Domains of GM1 Ganglioside

For the preparation of liposomes carrying such domains, the use of the molecular species of GM1 ganglioside carrying C_{20}-sphingosine is required, as formation of domains is dependent on the phospholipid environment. The main characteristics of these liposomes are the following: size 100 nm (1000 Å); shape monolamellar; gel to liquid-crystalline temperature transition (T_m) 41.5°C, determined by high-sensitivity differential scanning calorimetry. Therefore, the liposomes are in the physical state of gel up to this temperature, and this feature should be taken into accout anytime the physical state is important for the particular experiment to be performed.

For the preparation of these liposomes containing 10% molar ganglioside, mix 90 µL of the stock solution of DPPC with 100 µL of the stock solution of C_{20}-sphingosine GM1 ganglioside. The approximate final concentration of liposomes is 9 µmol of phospholipid/mL, 1 µmol of ganglioside/mL. The exact final concentration should be checked by phospholipid and sialic acid assay.

3.2.3. Liposomes Composed of SM, Containing Domains of Cholesterol and of GM1 Ganglioside

In these liposomes, distinct SM/cholesterol and SM/ganglioside domains coexist. The main characteristics of these liposomes are the following: size 100 nm (1000 Å); shape monolamellar; gel to liquid-crystalline temperature transition (T_m) 38°C, determined by high-sensitivity differential scanning

calorimetry. Therefore, the liposomes are in the physical state of gel up to this temperature, and this feature should be taken into account anytime the physical state is important for the particular experiment. For the preparation of these liposomes containing 10% molar ganglioside, mix 80 μL of the stock solution of SM with 100 μL of the stock solution of GM1 ganglioside and with 10 μL of the stock solution of cholesterol. The approximate final concentration of liposomes is 8 μmol of phospholipid/mL, 1 μmol of ganglioside/mL, 1 μmol of cholesterol/mL. The exact final concentration should be checked by phospholipid, cholesterol, and sialic acid assay. The reference temperature for this mixture important for the preparation of liposomes is 45°C.

3.2.4. Liposomes of DPPC, Containing GM1 Ganglioside Carrying C_{18}-Sphingosine, Not Forming Domains in This Phospholipid

For the preparation of these liposomes containing 10% molar ganglioside, 90 μL of the stock solution of DPPC is mixed with 100 μL of the stock solution of ganglioside. The approximate final concentration of liposomes is 9 μmol of phospholipid/mL, 1 μmol of ganglioside/mL. The exact final concentration should be checked by phospholipid and sialic acid assay. The reference temperature for this mixture, which will be important for the preparation of liposomes, is 45°C.

3.2.5. Liposomes Having Different Proportions Among the Components

Liposomes containing domains of GM1 or GD1a ganglioside, in proportions different from those described in the preceding in the standard procedure can be prepared, simply varying the amount of ganglioside in the standard recipe. Up to 20% molar percent GM1 ganglioside and up to 15% GD1a can be utilized. At higher molar percentages the stability of liposomes decreases while increasing their tendency to form mixed micelles instead of bilayers.

For SM/cholesterol/GM1 ganglioside liposomes, molar percentages can be varied up to 30% for cholesterol and up to 20% for ganglioside.

3.3. Preparation of Liposomes

3.3.1. Preparation of the Lipid Film

This step must be carried out the day before the actual preparation of liposomes. Usually, it is advisable to perform this first step in the afternoon and the subsequent steps on the following day.

1. Lipids are mixed in a vacuum-fitting test tube of 5 mL total volume, withdrawing proper amounts of each lipid from the stock solutions, in the proportions described in the preceding for the various types of liposomes.

2. Chloroform–methanol (2 : 1 v/v) is added to obtain a total volume of 400 μL. The solvent is slowly evaporated using a gentle stream of nitrogen, under the hood. During this step, the test tube must be kept inclined and continuously rotated. This can be achieved or rotating the test tube by hand or, better, fitting it to a rotating mechanical device (at about 60 rpm). Removal of solvent will produce the deposition of lipids as a film on the bottom and on the walls of the test tube. The removal must be slow (it should take about 5 min) in order to allow the proper mixing among the components. Alternatively, use a rotatory evaporator. In this case, be careful that no drops are ejected from the solution. Fit the tube to a lyophilizer and lyophilize overnight. Lyophilization overnight is recommended. If limited time is available, the lyophilization time can be reduced to 3 h, but this is not recommended. The presence of traces of solvent is deleterious for the assembly of domains.

3.3.2. Use of the Extruder

The extruder is assembled as specified by the manufacturer (Lipoprep). Two overlaying Nucleopore filters are placed in the extruder, handling them only with a flat-tip tweezers. The filters must be placed in the extruder maintaining the same orientation (up/down) as they are taken from their box.

The connected circulating bath is turned on and the temperature inside the extruder is set to reach is the reference temperature indicated for each type of liposomes. If the setting temperature is not known, the procedure is as follows: 1 mL of buffer, preheated at the reference temperature, is placed inside the extruder, then wait 10 min. The temperature of the buffer inside the extruder is measured until the reference temperature is reached. The circulating bath is run for about 30 min before proceeding with the following steps.

The extruder is loaded with 1.5 mL of distilled water using a Pasteur pipet. After 10 min the water is extruded. The pressure from the extruder is released and replaced. All the water at this point shall be removed. This is repeated two times.

To condition the filters, 1.5 mL of the buffer to be utilized for the preparation of liposomes needs to be extruded two times. Using a Pasteur pipet, 1.5 mL buffer is loaded. After a 10-min extrusion, pressure removal and repressurization are carried out. All the buffer will be removed after this step.

A tube containing about 3 mL of buffer, the tube containing the lipid film, and a glass pipet are placed in an oven at the temperature given below for the various types of liposomes. After thermostating for 20 min, 1 mL of buffer is withdrawn with the pipet. A propipet is used when hot. The buffer is added to the lipid film and vortex-mixed for 1 min. This is put in the oven for 5 min and vortex-mixed again for 1 min. The suspension is maintained at the reference temperature.

3.3.3. Extrusion of Liposomes

The extruder is loaded with the lipid suspension. Wait 5 min to ensure thermostatting. The suspension is extruded and collected in a test tube maintained at the reference temperature. The liposomes are extruded again, ten times, and collected in different test tubes each time, always thermostatted at the reference temperature.

4. Notes

Please consider the following points for a correct preparation of liposomes.

1. The final lipid concentration of liposomes is much lower than expected. Possible causes are: (a) the temperature of the solution in the extruder is lower than the reference temperature indicated for each type of liposomes (check the temperature inside the extruder as described in the preceding); (b) the concentration of stock solutions is not correct (assay the lipid concentration of stock solutions).
2. Liposomes are not coming out from the extruder. Possible causes are: (a) the temperature is not adequate (too low: adjust the temperature of the circulating bath); (b) the filters are clogged (raise the temperature and the pressure: if no effect is noticed, withdraw the lipid suspension from the extruder and replace the filters).
3. Liposomes are coming out too fast from the extruder or the lipid suspension is not becoming clearer after some extrusion steps. This occurs if the filters have been damaged. Commonly this is due to misuse of the Pasteur pipet used to load the extruder, or the tweezers used to handle the filters. Withdraw the lipid suspension from the extruder and replace the filters. Be careful not to touch the filters with the Pasteur pipet. Check the tweezers.

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