Scientific contribution

Preparation of Size-Controlled Unilamellar Lipid Vesicle Suspension from Lipid Powder : a Standard Operating Procedure within the Nanostructurome Methods Pipeline

Bar Laure [1,*], Lavrič Marta [2]

1. Faculty for Electrical Engineering, University of Ljubljana, Ljubljana 1000, Slovenia
2. Condensed Matter Physics Department, Jožef Stefan Institute, Ljubljana 1000, Slovenia

* Correspondence: Laure Bar; laure.bar@fe.uni-lj.si

Citation: Bar L, Lavrič M. Preparation of size-controlled unilamellar lipid vesicle suspen-sion from lipid powder : a Standard Operating Procedure with-in the Nanostructurome methods pipeline. Proceedings of Socratic Lectures. **2025**, 12(III), 11 – 17. https://doi.org/10.55295/PSL.12.2025.III2

Abstract:
Liposomes, owing to their high biocompatibility and ease of production and manipulation, are now largely employed as nanocarriers in drug delivery and as simplified cell-membrane models in biophysics for the study of their physical, structural, and nanomechanical properties. To ensure reliable results and sample stability, liposomes are most of the time unilamellar and of a specific size range. In this proceeding paper, some widely used process steps will be combined to form a complete methodology for forming unilamellar vesicles of defined diameter.

Keywords: Lipid vesicles; Extrusion; Vesicle characterization; SUV; LUV

Table of contents

1. Definitions

SUV: Small unilamellar vesicle
LUV: Large unilamellar vesicle
MLV: Multilamellar vesicle
DLS: Dynamic light scattering
DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine

2. Background

Liposomes are spherical structures composed of one or more phospholipid bilayers surrounding an aqueous core. These artificial lipid vesicles can be classified into different categories according to the size and number of bilayers (lamellarity) constituting the vesicles. Unilamellar vesicles are characterized as small (SUV) when the diameter is below 100 nm, large (LUV) when the diameter is between 100 nm and 1000 nm, or giant (GUV) when it is larger than 1000 nm. They are usually prepared from large multilamellar vesicles (MLV) corresponding to the onion-like structures created spontaneously when phospholipids are hydrated.
Liposomes are versatile in their composition, and easy to prepare. Their high biocompatibility and low immunogenicity allow them to be widely used as nanocarriers for many active molecules in drug delivery processes (Liu et al. 2022). For the latter, they indeed enhance drug efficacy by ensuring a better control over drug distribution and release (Lombardo et al. 2022). From a more fundamental point of view, liposomes can also be used as cell-membrane models of lower complexity for studying the inherent physical, structural, and nanomechanical properties of the different vesicular structures produced (Bar et al. 2023).

3. Purpose, Scope and Applicability

For reliable and reproducible results in experiments, lipid vesicles need to reach a certain degree of uniformity in terms of size and lamellarity, which can influence the behavior, the stability, and properties of the liposomes in experimental and biological systems. For instance, unilamellar vesicles of 50 nm to 200 nm are typically used in drug delivery, for optimizing the drug encapsulation yield and the liposome circulation half-time (Nsairat et al. 2022). For experimental research, this diameter range also offers optimal stability. Techniques such as the quartz crystal microbalance with dissipation monitoring benefit from this size range, as the vesicle layer is small enough to fit within the detection range yet large enough to generate strong signals.

For preparing lipid vesicles, several procedures can be followed, firstly for the way to form liposomes (such as the thin-film hydration method, solvent injection process, or the reverse-phase evaporation method), and secondly for uniformizing them to size-controlled LUVs (such as extrusion process, or sonication) (Patil et al. 2014, Šturm et al. 2021). In this paper, a detailed step-by-step methodology for forming LUVs will be explained.

4. Health and Safety Warning

The protocol proposed here implies the use of chloroform. Chloroform can cause serious health issues if not managed properly. Direct contact with the skin or eyes can cause irritation and burns. This chemical is also suspected of causing cancer. It is necessary to familiarize with the Material Safety Data Sheet providing information on handling, storage, and emergency measures.

5. Cautions

Use chloroform always under a fume hood and make sure that you wear personal protection equipment, including gloves, glasses, and laboratory coat. Store the container in a well ventilated place and follow all the proper procedures to prevent environmental contamination. In case of accidental exposure, immediately ask for medical assistance and contact the person in charge in your institution.
To prevent degradation of lipids, protect them from exposure to light, and store them at low temperature (fridge or freezer, depending on their specific safety data sheet).

6. Personnel Qualifications / Responsibilities

People performing the steps of this protocol should be familiar with the safety rules of a chemistry laboratory, which may include or even demand additional training from the employer.

7. Materials, Equipment and Supplies

Lipids, as well as the extrusion kit are purchased by Avanti Research™. Chemicals and salts must be of high purity, to minimize the presence and potential effects of impurities.

8. Computer Hardware and Software

No computer or software are necessary for performing this procedure.

9. Step by Step Procedure for Extruded Lipid Vesicle Formation

A well-established protocol for preparing unilamellar liposomes is presented in detail below, in the form of a *to do* list and illustrated in **Figure 1**. Some of the steps are accompanied by small useful tips or recommendations.

9.1. Formation of a Lipid Dried Film

The first operation consists of the dissolution of the lipids in an organic solvent. Lipids can be either supplied diluted in chloroform or in powder form.

- For lipids diluted in chloroform, calculate the volume needed according to the solution concentration and dispense this volume in a round-bottom flask. For lipids in powder form, dispense the mass needed in the round-bottom flask and add high-purity (> 99.9 %) chloroform until complete powder dissolution.

Always manipulate chloroform with a glass syringe. Using a plastic pipette tip could lead to some sample contamination due to plastic degradation.

- Under a fume hood, slowly dry the mixture with a continuous mild flow of nitrogen or argon to form lipid films. To improve the homogeneity of the dried

lipid film, the flask can be periodically rotated during the evaporation process. If the volume is more than 2-3 ml, use a rotary evaporator for working under vacuum and earn some time.

All residual organic solvent should be completely removed. The sample can be vented or kept under a vacuum chamber overnight to ensure the elimination of the last chloroform residues.

9.2. Lipid Film Hydration

The lipid film is now composed of lipid bilayers stacked over each other. Their hydration will lead to the bilayer detachment and formation of MLVs.

- Add fresh buffer so that the lipids are at a concentration of 2 mg/ml, and hydrate the sample by immersing the flask in a temperature-controlled water bath. The temperature of the latter is kept 15°C above the main lipid phase transition temperature for having vesicles in the liquid phase. Keep hydrating for 45 min under magnetic stirring.

In order to help the dried film detachment from the glass, the buffer used for the hydration can be pre-heated to a temperature at which the lipid is in the liquid phase. After adding the warm buffer into the flask, a short sonication of 1 min can also be performed before putting the sample into the water bath.

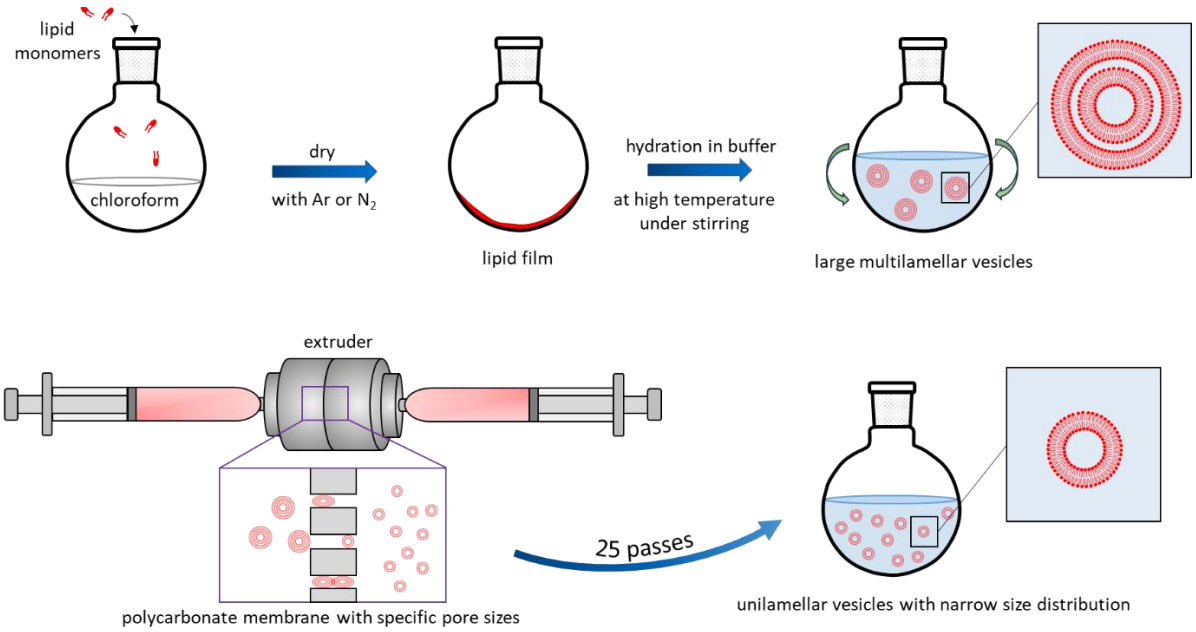

Figure 1. Scheme of the proposed protocol for forming SUVs or LUVs from lipids supplied in powder form.

9.3. MLVs Extrusion

After hydration, the MLVs solution may appear cloudy, but should be homogeneous.

- Prepare the extruder as explained in Section 10.
- Insert the lipid solution into one of the two syringes and perform a series of 25 passes.
- Dilute the extruded solution (now consisting of unilamellar vesicles) in buffer to obtain the desired working concentration.

The extrusion should be also done by keeping the lipids in the liquid phase.

10. Focus on Extrusion Material and its Preparation

Lipid vesicle extrusion (**Figure 2**) is a widely used process aiming at pressing hydrated MLV solutions through filters with specific pore sizes. This produces a uniform group of smaller vesicles (LUVs or SUVs), with their mean diameter corresponding to the pore size of the selected filters. Even though they operate in the same way, the commercial extruder sets can slightly vary in materials or features, such as the degree of automation and the regulation of temperature. The extrusion process explained here will be performed using the Avanti Research™ Mini-Extruder set. It is composed of Teflon membrane supports in which a polycarbonate membrane of specific pore size is sandwiched between two additional support filters. The solution coming from one syringe passes through this network to reach the opposite syringe. By doing this, the vesicles are broken and re-organized into smaller ones.

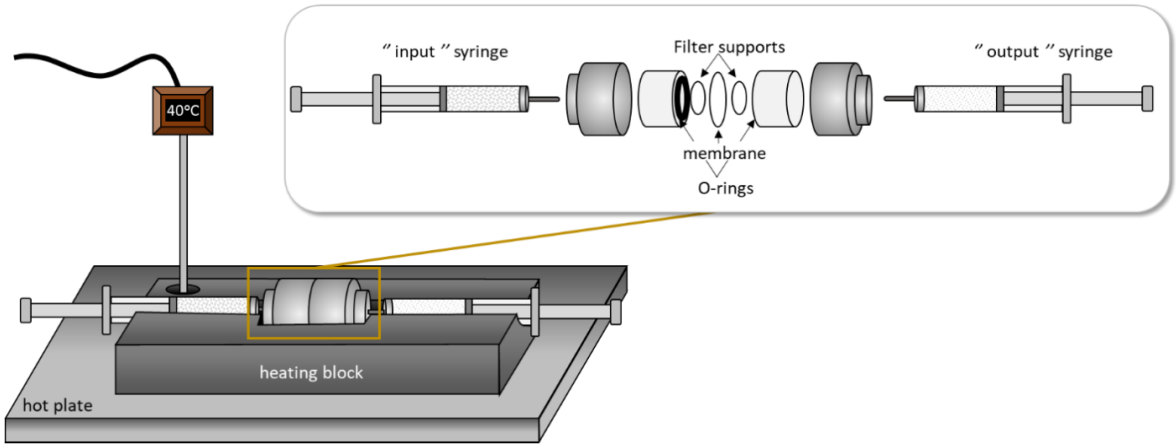

Figure 2. Scheme of an extruder setup and how it should be mounted and used for forming SUVs or LUVs.

For preparing the extruder, let's process as follows:

- Regulate the temperature of the extruder by placing the heating block and its associated thermometer on a hot plate and wait until reaching a stable temperature allowing the lipids to be in liquid phase.
- Assemble the membrane support system: use tweezers to gently handle the membranes. Filter supports can be pre-wetted with buffer before being placed, in order to reduce the dead volume.

Once the extruder is mounted, it is recommended to pass a full syringe of buffer through the setup to test if the system is well tightened and leakage-free. The buffer is then thrown away.

The extruder is now ready to be used as described in subsection 9.3.

Start the extrusion process from one syringe, and collect the solution from the syringe of the opposite side in order to retain the trapped impurities away from the suspension.

11. Data and Records Management

This section is not applicable for this SOP.

12. Related Protocols or SOPS

General instructions regarding the extrusion step can be found online on the Avanti Research website.

13. Quality Control and Quality Assurance

After extrusion, it is essential to assess the quality of the liposome suspension by checking the uniformity of the vesicles' mean diameter values and polydispersity. This can be done *via* different techniques, such as dynamic light scattering (DLS), nanoparticle tracking analysis, or transmission electron microscopy.

DLS is a non-destructive method allowing us to determine the particle size distribution by analysing the intensity variation of the light scattered within a colloidal solution, due to the Brownian motion of the particles in suspension. The Brownian motion describes the continuous collisions of particles within a suspension, which cause the particles' motion. The speed of the particles is related to their size: the smaller particles move faster than the larger ones. The Stokes-Einstein equation describing this relation highlights the influence of two parameters on the particle movement, and by consequence, the determination of the hydrodynamic diameter. These parameters are the working temperature and the viscosity of the dispersant (i.e. buffer).
An experiment can then be performed as follows:

- Switch on the DLS instrument several minutes before performing the measurement, to allow the laser light source enough time to warm up and reach a stable operating condition.
- Carefully set the parameter values needed in the software: the temperature, and the dispersant viscosity.
- Perform the measurement.

Figure 3 illustrates the size distribution of a 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) suspension before and after the extrusion in HEPES buffer. The non-extruded suspension displays two vesicle populations. The one representing 95% of the sample is characterized by a mean diameter of ~ 3.1 µm, corresponding to MLVs. Both the polydispersity index of 0.53 and the presence of a second smaller population indicate a high heterogeneity of the non-extruded suspension. On the contrary, once this solution was extruded using a membrane of 100 nm pore size, lipid vesicles attain an average diameter of 128 nm, and the polydispersity of the suspension is greatly reduced to 0.08. These data are in accordance with a homogeneous LUVs population. The final vesicle diameter is typically slightly higher than the membrane pore size, yet in the same range.

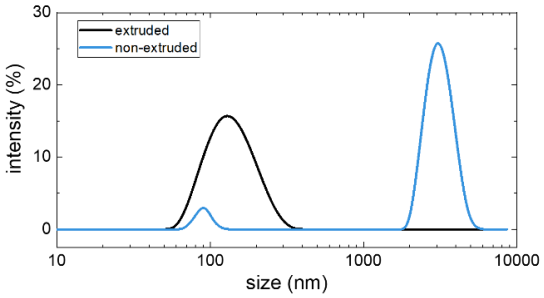

Figure 3. Size distribution plot obtained from the DLS measurement of a DMPC solution before and after extrusion with a 100 nm pore size membrane.

14. Conclusions

This paper presents a comprehensive step-by-step guide for the preparation of LUVs and SUVs utilizing widely used, standard processes. Key steps include the formation of a lipid film, the hydration of the film, and the extrusion of the resulting suspension. Due to its simplicity and high efficiency, this procedure proves exceptionally useful for applications in biochemical assays and membrane studies.

Funding: This research was funded by Slovenian Research Agency (ARIS) (grant numbers: J3-3066, J2-4447, P3-0388, J3-60063, and project Nanostructurome (according to a contract between ARIS and University of Ljubljana), and the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska–Curie Staff Exchange project "FarmEVs" (grant agreement no: 101131175). The views and opinions expressed in this publication are solely those of the authors and do not necessarily reflect those of the European Union. Neither the European Union nor the granting authority can be held responsible.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Liu P, Chen G, Zhang J. A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. Molecules 2022; 27:1372. https://doi.org/10.3390/molecules27041372
2. Lombardo D, Kiselev M.A. Methods of Liposomes Preparation: Formation and Control Factors of Versatile Nanocarriers for Biomedical and Nanomedicine Application. Pharmaceutics 2022; 1: 543. https://doi.org/10.3390/pharmaceutics14030543.
3. Bar L, Villanueva M.E, Neupane S, Cordoyiannis G, Losada-Pérez P. QCM-D Study of the Formation of Solid-Supported Artificial Lipid Membranes: State-of-the-Art, Recent Advances, and Perspectives. Physica status solidi (a) 2023; 220: 2370047. https://doi.org/10.1002/pssa.202370047.
4. Nsairat H, Khater D, Sayed U, Odeh F, Al Bawab A, Alshaer W. Liposomes: Structure, Composition, Types, and Clinical Applications. Heliyon 2022; 8: e09394. https://doi.org/10.1016/j.heliyon.2022.e09394.
5. Patil YP, Jadhav S. Novel methods for liposome preparation. Chem Phys Lipids 2014; 177: 8-18. https://doi.org/10.1016/j.chemphyslip.2013.10.011.
6. Šturm L, Poklar Ulrih N. Basic Methods for Preparation of Liposomes and Studying Their Interactions with Different Compounds, with the Emphasis on Polyphenols. Int J Mol Sci. 2021; 22: 6547. https://doi.org/10.3390/ijms22126547