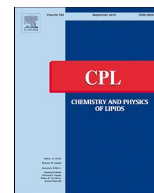
Chemistry and Physics of Lipids 212 (2018) 144–151

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip



Rapid single-step formation of liposomes by flow assisted stationary phase interdiffusion

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ARTICLE INFO

Keywords:
Liposome preparation
Stationary phase interdiffusion
Intrinsic vesicle diameter
Large unilamellar vesicles

ABSTRACT

Laboratory preparation of unilamellar liposomes often involves multiple steps carried out over several hours to achieve a monodisperse size distribution. Here, we present a methodology based on a recently introduced lipid self-assembly principle—stationary phase interdiffusion (SPI)—to prepare large unilamellar vesicles (LUVs) of a monodisperse population in a short period of about 10 min. The stationary interface between a lipid–ethanol phase and an aqueous phase is created by a density difference induced convective flow in a horizontal capillary. The average size of the liposomes, as expected from the SPI principle, is modulated only by the temperature and the type of lipids. Lipid concentration, ethanol content, pH of the aqueous phase, and the time duration of the experiment have little influence on the mean diameter of the vesicles. This simple methodology can be easily carried out with a capillary and a micro-needled syringe and provides a rapid production tool for researchers requiring reproducible liposome suspensions. Refined natural lipids, based on soy and egg lecithin mixtures, yield LUVs in the range 100–200 nm, suitable for drug delivery applications.

1. Introduction

Liposomes are widely used as model biomembranes as well as drug delivery vehicles (Lasic and Papahadjopoulos, 1996). The size and size distribution of liposomes are two important factors for *in vivo* applications. The size of liposomes influences the drug loading, biodistribution, drug clearance rate from the body, targeting efficacy to the specific organ, and the therapeutic efficiency (Dhand et al., 2014).

Liposomes can be prepared by several techniques, each of which affords the liposomes with special characteristics. Since the pioneering observation of Bangham and Horne (1964) more than half a century ago, research in the field of liposomes has seen a significant increase. A variety of techniques have been described in the literature for the preparation of liposomes (Patil and Jadhav, 2014), including the conventional methods: thin film hydration (Bangham and Horne, 1964), detergent depletion (Kagawa and Racker, 1971), ethanol injection (Batzri and Korn, 1973), and reverse phase evaporation (Szoka and Papahadjopoulos, 1978). Most of these techniques, even widely used today, usually require post-processing steps such as extrusion (Olson et al., 1979), or sonication (Szoka and Papahadjopoulos, 1980) to reduce the size, heterogeneity, and multi-lamellarity of the final liposome population.

The ethanol injection method has several advantages over other preparation methods; such as, instant formation of vesicles, possibility of preparing small sized vesicles without the use of any potentially harmful chemicals (ethanol is ICH class 3 residual solvent which is less toxic than ICH class 2 solvents such as chloroform, methanol, etc. (Grodowska and Parczewski, 2010)), simplicity, no special post treatments, and the possibility to scale up. In this method, the size of the liposomes is controlled by tuning the lipid concentration, injection rate of lipid/ethanol solution, lipid type, nature of aqueous media, rate of stirring, volume of ethanol injected (Kremer et al., 1977; Pons et al., 1993) and the operating temperature (Justo and Moraes, 2011). However, one primary disadvantage of this method is the polydisperse population and formation of multilamellar vesicles, mainly due to the turbulent mixing at the ethanol–water interface. For better controlling the mixing conditions, this method has been improvised in a microfluidic geometry under laminar flow conditions (Jahn et al., 2004), where unilamellar monodisperse liposomes can be directly obtained. However, even here there is a viscosity gradient induced fluid mechanical instability that can lead to other factors influencing the liposome formation, making it hard to obtain a predictive model for the size of liposomes (Phapal and Sunthar, 2013).

Recently, we have shown, in a special case of the ethanol–lipid–water system, it is possible to obtain a theoretical estimate of the liposome diameter from the Helfrich's free energy of liposomes (Helfrich, 1974; Phapal et al., 2017). The principle, called Stationary Phase Interdifusion (SPI), involves contacting the lipid–ethanol phase with the

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https://doi.org/10.1016/j.chemphyslip.2018.01.007
Received 30 September 2017; Received in revised form 28 December 2017; Accepted 16 January 2018
Available online 31 January 2018
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aqueous phase in a stationary manner with the mixing dominated by molecular diffusion. There is no injection of ethanol or other laminar co-flows. The size of liposomes is dependent only on the lipid type and the solvent temperature. None of the other parameters such as lipid concentration, ethanol–water ratio, or other macroscopic flows (present in other systems) influences the diameter of liposomes. This makes the method robust and predictable. Liposomes can be prepared in about 6 h time.

In the present work, we implement the SPI principle in a horizontal capillary system (the liposomes were earlier formed in a vertical cuvette (Phapal et al., 2017), and find a remarkable reduction in the time of preparation. The procedure is described in Section 2, the influence of various commonly known parameters is presented in Section 3, and a brief discussion on the results is given in Section 4.

2. Materials and methods

2.1. Materials

Lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in powder form were gifted by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol in a powder form and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) in solution form (lipid in chloroform) were procured from Avanti Polar Lipids (Alabaster, AL, USA). Two commercial lipids Lipova E120 (purified egg lecithin with minimum 93% phospholipid) and Leciva S90 (purified soy lecithin with minimum 90% PC) were gifted by VAV Life Sciences (Mumbai, India). Ethanol (AR grade) was used as supplied from Merck (Darmstadt, Germany). Sodium salt of fluorescein (dye) was purchased from HiMedia (India). Milli-Q water was used as the aqueous solvent. To avoid large extraneous particles, the milli-Q water was filtered through a 200 nm filter paper. Long SS needles (25G, 10 cm length) were purchased from Victor-G. & Company (Kanpur, India).

2.2. Preparation of lipid solution

The lipid solution is prepared in ethanol. The lipids in powdered form (DMPC, DOPC, DPPC, Lipova E120, Leciva S90, and cholesterol) are directly added to an appropriate volume of ethanol. In the case of lipids in a solution form (SOPC in chloroform), it is placed in a round bottom flask (RBF) and the organic solvent is evaporated under reduced pressure inside a rotary evaporator (Roteva Equitron, Medica Instruments, Mumbai, India) at a speed of 150 rpm for 5 min and maintained at 55 °C. The RBF is kept in a vacuum desiccator overnight to remove traces of chloroform and after that ethanol is added to the dried lipid.

2.3. Protocol for the preparation of liposomes

A schematic diagram of the preparation steps is shown in Fig. 1. The main components are: (a) a one-end sealed glass capillary tube (ID: 1 mm; OD: 1.4 mm; length: 100 mm), (b) three 10 cm long 25G SS needles (served by glass syringes), one each for the aqueous phase, the lipid phase, and for removing the air gap, (c) sealants (paraffin film/wax). As illustrated in the schematic: (A) The aqueous phase is first filled up to about 4 cm. (B) The lipid phase containing a mixture of lipid–ethanol and water (lipid–ethanol at 10 mg/ml is premixed with water at 50%, v/v) is taken in another syringe and filled up to 4 cm leaving about 5 mm air gap between the two phases. (C) The two miscible phases are brought into contact by gently withdrawing the air through the third syringe. While removing the air gap, care is taken to avoid any mechanical disturbance of liquid–liquid interface; otherwise, a multi-modal size distribution of liposomes may be observed. (D) After creating the interface, the needle is gently taken out of the capillary and the open end of the capillary is sealed with a sealant to reduce the evaporation of ethanol. The capillary is then placed horizontally in an incubator for 45–60 min, at a temperature above the phase transition temperature T_m of the lipid bilayer-membrane (e.g., T_m = 24 °C for DMPC). A video demonstration of this procedure is available as a supplementary data in Appendix A.

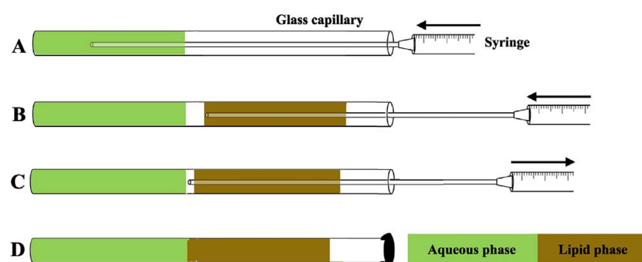

Fig. 1. Schematic illustration of the experimental procedure. (A) The aqueous phase is filled in the capillary at the sealed end. (B) The lipid phase is filled leaving a small air gap between the phases. (C) The air gap between the two phases is removed with the help of a thin, long needle to create the interface between the liquids. (D) The open end is sealed with the help of a sealant.

2.3.1. Limitation of ethanol concentration

The above procedure can be carried out for varying volumes of the aqueous and lipid phases and also of water in the lipid phase. However, it is essential to ensure that the concentration of ethanol in the entire liquid volume (after complete mixing) does not exceed 35% (v/v). It is known from experiments (Mou et al., 1994) and molecular dynamics simulation (Gurtovenko and Anwar, 2009) that beyond a threshold ethanol concentration (29.4% (v/v) from experiments and 30.5% (v/v) from simulations) the vesicles are no longer stable and disintegrate to form small globular shapes. In the present setup, ethanol is limited to 25% (v/v) of total liquid in the capillary.

2.4. Liposome characterization methods

2.4.1. Dynamic light scattering

Dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern), also known as photon correlation spectroscopy (PCS) (Ostrowsky, 1993), is used for the analysis of liposome size and size distribution (polydispersity). Samples from the capillary setup are diluted with milli-Q water (80–100 folds for 10 mg/ml lipid/ethanol solution), to get a reliable particle count rate. The mean diameter of the liposomes and its distribution are computed using the software (DTS version 7.11) provided with the instrument. The mean size is taken as the *Z*-averaged value and the size distribution is expressed in terms of the polydispersity index (PDI), both obtained from the software. Three readings are obtained for each sample, and each experiment is repeated at least three times.

2.4.2. Microscopic observations: morphology and lamellarity

The size distribution of liposomes is also obtained using scanning electron microscopy (SEM, Model- JSM 7600F, JEOL, MA, USA) in the cryogenic mode (Cryo-SEM). For this analysis, a small drop of the liposome suspension is placed on a sample holder and plunged into slush nitrogen (−190 °C) for freezing the sample. The sample is then transferred into the SEM chamber, where the frozen sample is sublimed for 5 min before capturing the images. Transmission electron microscopy (TEM, Model-JEM 2100F, JEOL, Tokyo, Japan) is used in cryogenic mode to observe the lamellarity of the vesicle membranes. Cryo-TEM analysis involves placing a drop of the liposome suspension (about 5 μl) onto a TEM grid (Lacey carbon film coated with copper grid) with the help of a micropipette. The grid is then blotted with a Whatman filter paper in order to remove any excess solution, and quickly plunged into liquid ethane (−180 °C) by the Vitrobot plunge freezer (FEI, USA). Thereafter, the vitrified sample, under liquid nitrogen, is transferred to

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