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Preparation of liposomes: A novel application of microengineered membranes–From laboratory scale to large scale

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ABSTRACT

A novel ethanol injection method using microengineered nickel membrane was employed to produce POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and Lipoid® E80 liposomes at different production scales. A stirred cell device was used to produce 73 ml of the liposomal suspension and the product volume was then increased by a factor of 8 at the same transmembrane flux (140 l m^{-2} h^{-1}), volume ratio of the aqueous to organic phase (4.5) and peak shear stress on the membrane surface (2.7 Pa). Two different strategies for shear control on the membrane surface have been used in the scaled-up versions of the process: a cross flow recirculation of the aqueous phase across the membrane surface and low frequency oscillation of the membrane surface (~40 Hz) in a direction normal to the flow of the injected organic phase. Using the same membrane with a pore size of 5 μm and pore spacing of 200 μm in all devices, the size of the POPC liposomes produced in all three membrane systems was highly consistent (80–86 nm) and the coefficient of variation ranged between 26 and 36%. The smallest and most uniform liposomal nanoparticles were produced in a novel oscillating membrane system. The mean vesicle size increased with increasing the pore size of the membrane and the injection time. An increase in the vesicle size over time was caused by deposition of newly formed phospholipid fragments onto the surface of the vesicles already formed in the suspension and this increase was most pronounced for the cross flow system, due to long recirculation time. The final vesicle size in all membrane systems was suitable for their use as drug carriers in pharmaceutical formulations.

1. Introduction

Liposomes are versatile drug carrier systems that can be tailor-made to accommodate a large variety of drugs for a wide range of therapies. Both lipophilic and hydrophilic drugs can be incorporated in liposomes, within the phospholipid bilayer and in the aqueous core, respectively [1]. The behaviour of liposomes *in vivo* and *in vitro* can be controlled by selecting the proper characteristics such as vesicle size, number of bilayers, bilayer fluidity, charge and hydrophilicity of the external surface, and the type of targeting molecules attached to the bilayer surface [2]. The applications of lipid vesicles are determined by their properties, which depend on molecular and physicochemical parameters as well as on the method of liposome preparation [3]. Therefore, a well-characterized methodology for liposome manufacture with validated operating procedures is the main requirement for producing liposomal populations with acceptable reproducibility and appropriate for the intended use.

Liposomal preparations can be manufactured using a wide variety of methods such as thin film hydration, reversed-phase evaporation, detergent dialysis, and solvent injection [4]. The major challenge in liposome production is still large scale production. Indeed, most of the described preparation techniques are not suitable for scaling up from the laboratory level to the industrial production, due to their complexity and a low reproducibility and predictability of the preparations obtained. A lack of predictability of product quality may be attributed to empirical methods traditionally employed for the design of lipid-based delivery systems [5]. Thus, there is a strong need to improve traditional manufacturing techniques, leaving behind those poorly characterizable methods, based on small batch sizes.

The ethanol injection method can be used for liposome production at large scale. In this process, an ethanolic solution of the lipid mixture is dispersed into an aqueous solution through fast injection. From the manufacturing point of view, this technique does fulfil the need for a rapid, simple, easily scalable and safe preparation technique. Also, this method does not promote degradation or

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oxidative alterations either in the lipid mixture or in active agents to be encapsulated [6].

Membrane dispersion, which is considered as an improvement of the ethanol injection technique, is a new method of producing liposomes of predetermined size. It involves mixing of two miscible liquids (the organic and aqueous phase) by injecting the organic phase through a microporous membrane into the aqueous phase. It is similar to membrane emulsification [7,8], which involves the injection of one liquid (the dispersed phase) into another immiscible liquid (the continuous phase) through a microporous membrane [9,10]. Micro-engineered membranes, which have a perfect hexagonal array of uniform pores, allow a much more uniform and controllable injection of lipid-containing organic phase into an aqueous phase. Thus, their use enables a better control over diffusive mixing at the liquid/membrane interface where the lipids self-assemble into vesicles. This may provide fine control of liposome size distribution and make easier the extrapolation of the results for an industrial large scale production. The shear stress at the membrane surface can be controlled by [11]: (i) stirring the continuous phase using a paddle stirrer (Fig. 1a); (ii) cross flow of the continuous phase along the membrane surface (Fig. 1b); (iii) vibrating (oscillating) the membrane in the continuous phase (Fig. 1c).

Recent studies [10,12] were focused on the fabrication of liposomes using Shirasu Porous Glass (SPG) membrane. It was found that the vesicle size decreased with a decrease in the transmembrane flux and phospholipid concentration in the organic phase and with an increase in the aqueous to organic phase ratio and the shear stress on the membrane surface. Despite all the information provided in the literature regarding the effect of different operating and process conditions on vesicle characteristics [13–15], there is a lack of information regarding scale-up of liposomes production.

The aim of this study was to evaluate the scale-up of liposome production by a factor of 8 and beyond using novel ethanol injection method with microengineered membrane. For a small-scale production, a laboratory stirred cell was used, composed of a rotating stirrer above a flat disc membrane. For large scale production, two different methods were used: (a) recirculation of the continuous phase in cross flow along the membrane surface, and (b) oscillation of the membrane surface in a direction normal to the flow of the injected phase.

2. Materials and methods

2.1. Reagents

Phospholipids used in this study were POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and Lipoid® E80 (obtained from egg yolk lecithin and containing 82% of phosphatidyl-choline and 9% of phosphatidyl-ethanolamine), both purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol and phosphotungstic acid were supplied by Sigma–Aldrich Chemicals (Saint Quentin Fallavier, France). 95% analytical-grade ethanol was supplied by Fisher Scientific (United Kingdom) and used as such, without further purification. Ultra-pure water was obtained from a Millipore Synergy® system (Ultrapure Water System, Millipore).

2.2. Membranes

The membranes used were nickel microengineered membranes containing uniform cylindrical pores arranged in a hexagonal array with a diameter of 5 or 20 μm and pore spacing of 200 μm. The membranes were fabricated by the UV-LIGA process, which involves galvanic deposition of nickel onto a template formed by photolithography [16]. All membranes were supplied by Micropore Technologies Ltd. (Hatton, Derbyshire, United Kingdom).

2.3. Experimental equipment

Schematic illustration of the equipment used is presented in Fig. 2.

2.3.1. Stirred cell device

A Dispersion Cell was supplied by Micropore Technologies Ltd. (Hatton, Derbyshire, UK). This device uses a 24 V DC motor (INSTEK model PR 3060) to drive a paddle-blade stirrer at an adjustable speed controlled by the applied voltage. An effective diameter of the membrane fitted at the bottom of the cell was 3.3 cm and a membrane area was 8.55 cm^2. The organic phase was injected through the membrane using a peristaltic pump (Watson Marlow 101U, Cornwall, UK).

2.3.2. Cross flow system

Cross flow module (Micropore Technologies Ltd.) was composed of 4 separate disk membranes, each with a diameter of 7 mm, so the total membrane surface area was 1.54 cm^2. The cross flow channel was 20 mm wide and 1 mm high. A syringe pump (Havard Appartus 11 Plus) was used to inject the organic phase through the membranes and a peristaltic pump (Watson Matlow 603s, Cornwall, UK) was used to recycle the aqueous phase between the module and an aqueous phase tank.

2.3.3. Oscillating membrane system

This system was also supplied by Micropore Technologies Ltd. The membrane was composed of 2 foils rolled in the form of a ring with a diameter of 30 mm and a length of 20 mm. The membrane had an area of 34.1 cm^2 and was attached to the injection manifold to which an accelerometer was fixed. The accelerometer (PCB Piezotronics model M352C65) was connected to a National Instruments Analogue to Digital Converter (N1 Edaq-9172) which was interfaced to a LabView executable program running on a computer. The information provided by the program from the accelerometer was the frequency and the amplitude of the oscillations, the amplitude being determined by the direction of the travel and the frequency was deduced from the acceleration measurement. The oscillation signal was provided by an audio generator (Rapid Electronics), which fed a power amplifier driving the electromechanical oscillator on which the inlet manifold was mounted. The injection manifold had internal drillings to allow the passage of the organic phase by a syringe pump (Harvard Apparatus 11 Plus).

2.4. Experimental procedure and shear stress calculation

The organic phase was composed of 20 mg ml^{-1} of phospholipids and 5 mg ml^{-1} of cholesterol (used as a stabilizer) dissolved in ethanol.

2.4.1. Stirred cell system

The cell was filled with 60 ml of ultrapure water and 13 ml of the organic phase was injected through the membrane at 2 ml min^{-1} to achieve a final volume ratio of the aqueous to organic phase of 4.5. The organic phase flux, J, was given by:

$$J = \frac{Q_o}{A} \quad (1)$$

where Q_o is the volume flow rate of the organic phase and A is the membrane area. The organic phase flux was 140 l m^{-2} h^{-1}, calculated from Eq. (1), and the stirrer speed was 600 rpm. Previous studies in Dispersion Cell [17,18] have shown that a shear stress is