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LARGE-SCALE PRODUCTION OF LIPOSOMES BY A MICROFLUIDIZER

by

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Abstract

Liposomes can be produced by a variety of techniques such as sonication, high-pressure homogenization, detergent dialysis, reverse-phase evaporation and ether injection. The Microfluidizer is a high-pressure homogenizer which is capable of making small size multilamellar vesicles. The mean vesicle size of the liposome reduced drastically from 0.64 micron to 0.16 micron after three passes through the Microfluidizer. Additional three passes did not reduce the size further. The polydispersity reduced about 1.25% per pass. The ability to scale up the liposome production from 100 ml to 4000 ml (representing minimal batch sizes for the lab scale and the production scale Microfluidizer) was investigated and found to be satisfactory.

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Introduction

Liposomes are vesicles which form spontaneously when appropriate composition of phospholipids are hydrated in an aqueous media. Liposomes can be produced by a variety of techniques. Small unilamellar vesicles (SUVs) can be prepared by sonication (1,2) or French Press (3) techniques. Large unilamellar vesicles (LUVs) can be made by dilution of organic solvents (4,5,6) or detergent dialysis (7,8,9). Multilamellar vesicles (MLVs) can be made by membrane extrusion (10,11) or homogenization with Microfluidizer (12). Extrusion can also produce unilamellar vesicles depending upon the selection of proper filter pore size. Many of these techniques are limited to laboratory-scale preparation.

Microfluidization is a relatively new technique which utilizes the force of two streams of liposome suspension colliding with each other under high pressure to reduce the vesicle size. Figure 1 shows a diagram of various components and direction of product flow in the Microfluidizer. Uniformly hydrated phospholipid suspension (unsized liposomer) is transferred to the reservoir. The liposome suspension is pumped under high pressure through the interaction chamber. In the interaction chamber, the suspension is divided into two streams and then recombined at high velocity to produce smaller and more uniformly sized liposome vesicles.

Water soluble drug, metaproterenol sulfate was selected to entrap in liposome preparation. Efficacy studies in our laboratories indicated that metaproterenol sulfate liposome preparation is pharmacologically active. Further , liposomally entrapped metaproterenol sulfate reduces the tachycardia side effect, which is frequently associated with the therapeutic use of this drug. Process scale-up for this preparation was required to prepare metaproterenol sulfate liposome in large batch sizes.

The ease to scale up the production is an important consideration in selecting production equipment. This study was undertaken to investigate the scalability of the Microfluidizer and to characterize the liposomes produced by the Microfluidizer.

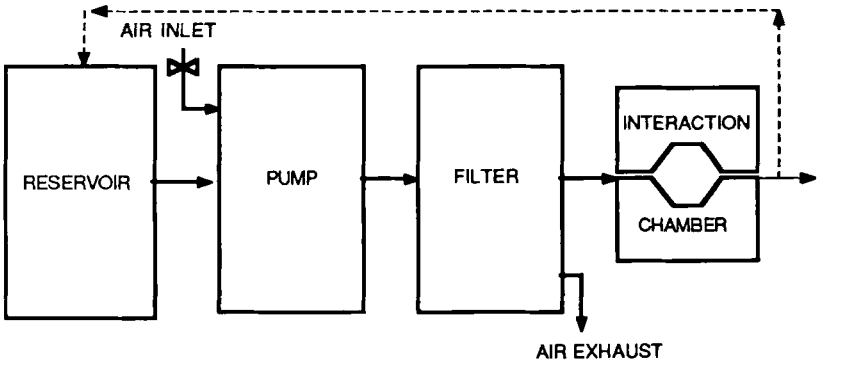

FIGURE 1

Block Diagram of Different Components and Flow of Product in Microfluidizer. Dashed line indicates possible product recycling through the system.

Materials and Methods

Materials

Injectable grade egg phosphatide (phosphatidyl choline 65% and phosphatidyl ethanolamine 17%) and egg phosphatidyl choline, 99% (Asahi Chemical Industry Company, Ltd., Japan), egg pohosphatidyl glycerol, 95% (Avanti Polar Lipids, Inc., Birmingham, AL) and cholesterol (Croda, Inc., Chatham, NJ) were purchased and tested for purity before use. dl-alpha tocopherol, disodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and edetate disodium were obtained from Sigma Chemicals (St. Louis, MO). All chemicals were used as received.

Formulation A consisted of 74.6% egg phosphatide, 24.8% cholesterol, and 0.7% of dl-alpha-tocopherol, while formulation B consisted of 62.1% egg phosphatidyl choline, 12.4% egg phosphatidyl glycerol, 24.8% cholesterol, and 0.7% dl-alpha-tocopherol. In both cases, all ingredients were dissolved in chloroform and dried extensively under reduced pressure in a rotary evaporator. An aqueous solution of drug (metaproterenol sulfate, 125 mg/ml) in

a hydrated lipid suspension were processed through a high shear mixer to break up the lumps. Uniformly hydrated lipid suspensions (unsized liposomes) were then processed through the Microfluidizer. The liposomes processed through Microfluidizer are called "liposome concentrate".

Equipment

A laboratory scale Microfluidizer (M-110) and a Production Scale Unit (M-210) (Microfluidics Corp., Newton, MA) were used in the study. The process variables studied were operating pressure, number of cycles through the Microfluidizer, and different sets of interaction/back pressure chambers. Different "interaction chambers" offer different channel lumens and, in turn, the velocities at which the streams combine.

Methods

Determination of Liposome Size Distribution

Liposome size distribution was determined by dynamic light scattering analysis, using Nicomp 270 submicron particle sizer equipped with a Helium Neon Laser. This technique is also referred to as quasi-elastic light scattering or photon correlation spectroscopy. Nicomp 270 employs digital autocorrelator to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in the solution. The measured diffusion coefficient is used in the Stokes-Einstein relation to obtain the average vesicle radius and hence, the mean diameter of liposome vesicles.

Electron Microscopy

The liposome concentrate samples were suspended in an aqueous solution of 20 - 30% glycerol. The suspension was then frozen in Freon 22 and fractured at 10^{-6} to 10^{-7} torr vacuum. The freeze-fracture was performed on a Balzer Freeze-Etching System BAF

400D. The micrographs were obtained by JEM-1200 Ex Electron Microscope. Details of this technique can be found elsewhere (13).

Quantitation of Encapsulated Drug

One gram of the sample (liposome concentrate) was diluted with 50 ml volume of normal saline and mixed well. The diluted samples were analyzed for total metaproterenol sulfate either by "detergent method" or by "extraction method". An aliquot of the diluted suspension was centrifuged at 100,000 rpm (4°C) for 30 minutes (Ultracentrifuge, Model TL100, Beckman Instruments, Inc., Palo Alto, CA). The supernatant and pellet were separated. The pellet was reconstituted in known volume of normal saline. Equal portions of reconstituted pellet (or diluted liposome concentrate) and 1% (w/v) dimethyldodecylamine oxide (DDAO) in tris buffer (ph 7.4) were mixed. The resulting clear solution was analyzed by HPLC for metaproterenol sulfate concentration in pellet. Another aliquot of the diluted liposome was treated with DDAO and analyzed for drug in the liposome concentrate.

In some experiments, metaproterenol sulfate was extracted into the aqueous phase from liposomes. In this technique, either the liposome concentrate or liposome paste was first diluted 1:10 with normal saline. The diluted suspension was extracted with a chloroform-methanol (1:1) solution. The lipids were solubilized into chloroform-methanol layer and the water soluble drug was extracted into the aqueous layer. The aqueous layer was assayed for drug concentration.

The percentage of drug encapsulated was calculated by the ratio of the drug in the pellet to the drug in the liposome concentrate.

Drug Release Rate

One gram of liposome concentrate is diluted 1:10 with normal saline and aliquots were dispensed into 5 ml vials, sealed, and placed into an end-to-end tumbler device (Van-Kel

Industries, Edison, NJ). These samples were rotated at 75 rpm for various lengths of time. At the indicated time intervals, the samples were collected. Each suspension was centrifuged at 100,000 rpm for 30 minutes at 4°C. The collected supernatant was assayed for drug concentration. The pellet was extracted with chloroform-methanol solution. The aqueous layer was assayed for drug concentration by HPLC. Time course of change in drug concentration in supernatant was calculated.

HPLC Analysis of Metaproterenol Sulfate

Metaproterenol sulfate content of various samples was assayed by HPLC after samples were prepared by extraction or DDAO method. The chromatography system (IBM Instruments, Danbury, CT) consisted of an IBM model LC/9560 and a variable wavelength UV detector (LC/9563) equipped with an injection valve of 20 microliter loop and an automatic sample LC/9505. The output of the detector signal is fed to an IBM 9000 computer system to generate the chromatogram. A C-18 (10 micron particle diameter, 4.5 mm x 250 mm) IBM analytical column was used. The flow rate was 1 ml/min. Detector wavelength was set at 276 nm. The mobile phase consisted of 70% phosphate buffer and 30% methanol.

Results and Discussion

The effects of the number of passes on the vesicle size and drug encapsulation are shown in Figure 2. Formulation B and Microfluidizer M-110 were used. As can be seen, the Microfluidizer drastically reduced the vesicle size from 0.64 to 0.24 micron after the first pass. The vesicle size gradually reduced further to 0.18 micron after the second pass and then stabilized at about 0.16 micron after three passes. Drug encapsulation also reduced sharply after the first pass and then slightly reduced thereafter. The encapsulation efficiency appears to be a function of the vesicle size (Figure 3). The larger the diameter of the vesicle, the more captured volume per surface area and, therefore, the higher the encapsulation efficiency per gram of phospholipids. Figure 4 shows the effect of the

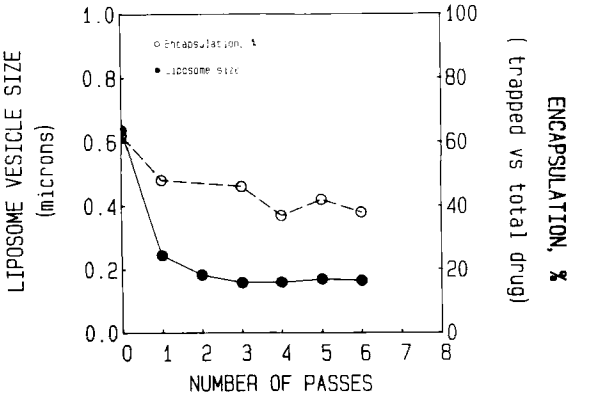

FIGURE 2

Liposome Vesicle Size and Drug Encapsulation vs. Number of Passes Through the Microfluidizer M-110, Processed at 3000 psi. Key: • % encapsulation; o vesicle size.

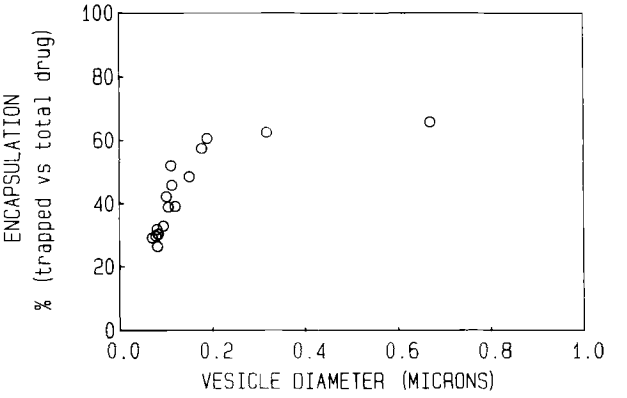

FIGURE 3

Effect of Liposome Diameter on Drug Encapsulation.

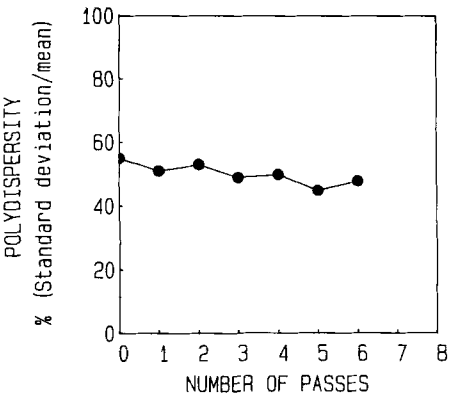

FIGURE 4

Liposome Polydispersity vs. Number of Passes Through the Microfluidizer M-110, Processed at 3000 psi.

number of passes on the polydispersity (i.e. the standard deviation divided by the mean) of the size distribution. The polydisperity reduced about 1.25% per pass. From the above results, it is concluded that three passes are adequate to achieve an optimal size reduction. The remaining studies are, therefore, all conducted at three passes.

Tables 1 and 2 showed the effects of interaction chamber size and processing pressure on the vesicle size and drug encapsulation of the liposomes produced by Microfluidizer M-110 and M-210, respectively. Formulation A was used because the phosphatidylglycerol required in Formulation B was too costly for the minimal batch size required by Microfluidizer M-210. It can be seen from Tables 1 and 2 that the higher the processing pressure, the smaller the vesicle size and the lower the drug encapsulation. It can also be seen from Table 1 that the larger the interaction chamber, the bigger the vesicle size it produced at a given pressure.

Figure 5 shows the electron micrographs of liposomes before and after microfluidization. Before microfluidization, the liposomes are consisted of multilamellar vesicles, as shown in

TABLE 1

Effects of Size of Interaction Chamber and Processing Pressure on the Vesicle Size and Drug Encapsulation. Formulation B and Microfluidizer M-110 Were Used.

Interaction Chamber ID Nos.*	Chamber Operating Pressure (PSIG)	Vesicle Size, Micron Mean (Std. Deviation)	Encapsulation (% Trapped)
	Before Sizing	0.637 (0.350)	69.6
A/B	11,000	0.070 (0.019)	29.1
A/B	8,000	0.080 (0.023)	31.8
D/E	11,000	0.083 (0.027)	30.3
D/E	8,000	0.105 (0.034)	38.9

* Chamber ID Nos. are in the order to increasing chamber size. Smallest chamber is designated as A while the largest chamber is denoted as E.

TABLE 2

Effects of Processing Pressure and Number of Passes on the Vesicle Size and Drug Encapsulation. Formulation A and Microfluidizer M-210 Were Used

Interaction Chamber ID#	Chamber Operating Pressure (PSIG)	No. of Passes	Vesicle Diameter, Micron Mean (Polydispersity*) Immediately	After 2 Days	Encapsulation (% Trapped)
	Before Sizing	--	--	0.637 (55)	69.6
D/E/F	3,000	1	0.189 (45)	0.255 (56)	65.2
	3,000	2	0.187 (46)	0.127 (51)	70.4
	3,000	3	0.165 (42)	0.178 (45)	74.6
	6,000	1	0.161 (40)	0.208 (48)	67.2
	6,000	2	0.137 (38)	0.151 (41)	67.7
	6,000	3	0.132 (36)	0.159 (43)	61.8
	9,000	1	0.158 (40)	0.165 (45)	60.9
	9,000	2	0.149 (38)	0.158 (42)	57.9
	9,000	3	0.154 (39)	0.150 (41)	57.8

* Polydispersity = $\frac{\text{standard deviation}}{\text{mean}} \times 1000$

Figure 5A. After microfluidization, the vesicles became more uniform in size and consisted of predominantly one or two layer vesicles, as shown in Figure 5B.

Figure 6 represents the drug release profiles of liposomes produced by Microfluidizer M-210 and M-110. The top curve in the figure is the drug release profile of liposomes produced by Microfluidizer M-110 and the bottom curve is that of the Microfluidizer

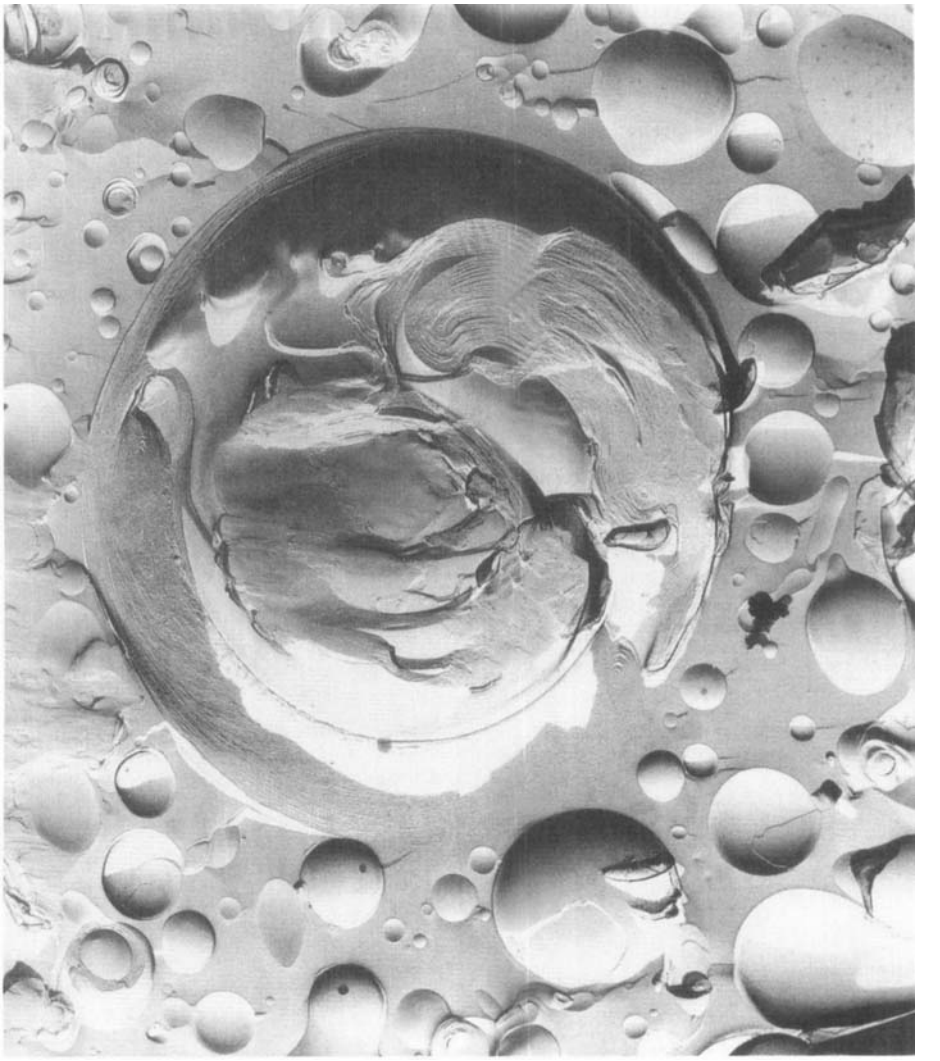

FIGURE 5(A)

Freeze-Fracture Electronmicrographs of Liposomes. (A) Hydrated Liposomes Before Microfluidization; (B) Microfluidized Liposomes Preparation. Bar in the figure represents 0.5 micrometers.

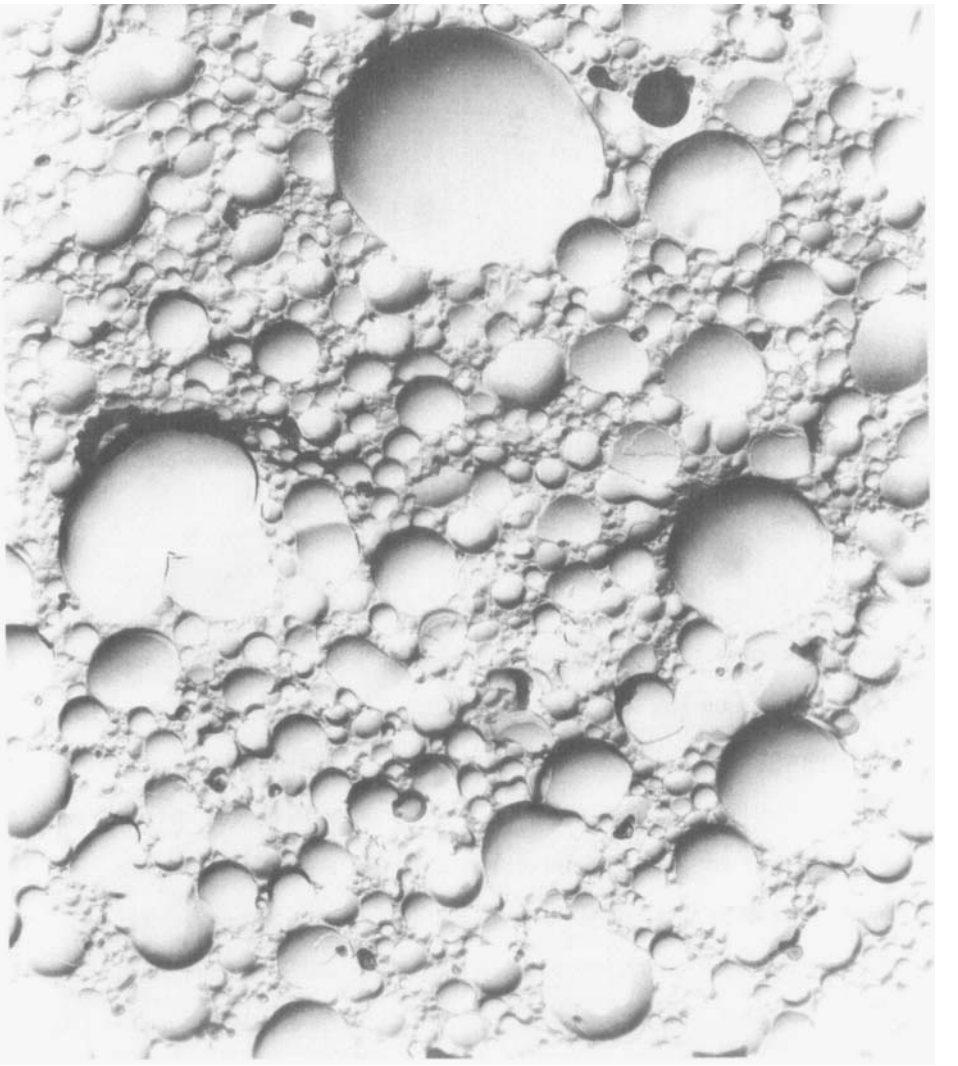

FIGURE 5(B)

M-210. The two release profiles are very similar except the amount of free drug (as indicated by the zero time data). The difference in the free drug content is due to the difference in encapsulation efficency between the M-110 and M-210 microfluidizers, as can be seen from the comparison of Tables 1 and 2. The encapsulation efficiency can be further correlated to vesicle size. Figure 3 shows the average vesicle diameter of

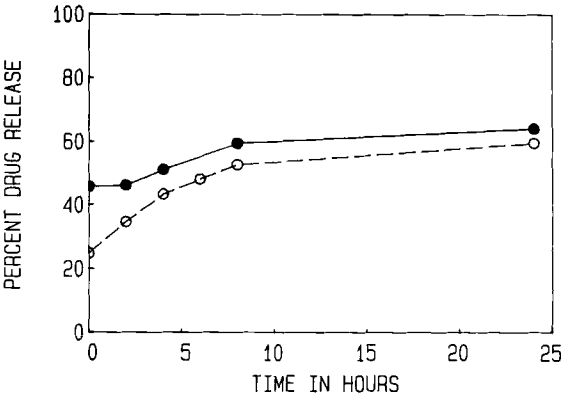

FIGURE 6

Drug Release Profile of Liposomes Prepared on Microfluidizer M-110 and M-210 at 3000 psi. Key: • processed on M-110; o processed on M-210.

liposomes and corresponding encapsulation efficiencies. As the vesicle diameter reduced from 0.2 to 0.1 micron size, the encapsulation efficiency sharply reduced from 65 to 25 percent. However, the vesicle diameters of 0.2 microns and larger maintained a better-than-60% encapsulation. It appears that the encapsulation efficiency is directly related to the captured volume per mole of lipid. For example, the captured volume of a sphere of 0.2 micron diameter is eight times the captured volume of a sphere of 0.1 micron in diameter. However, the surface area of a 0.2 micron sphere is only four times the surface of a 0.1 micron sphere. If one mole of lipid can make X number of 0.2 micron vesicles, then the same amount of lipid can only make 4X of 0.1 micron vesicles -- 50% short of the 8X number of 0.1 micron vesicles needed to give an equal captured volume to the X number of 0.2 micron vesicles. Therefore, for a given quantity of liposomes, the larger the vesicle size the higher the encapsulation. Further, it was found that the vesicle size is a function of the size of the interaction chamber and the operating pressure. In this study, the authors achieved better encapsulation efficiencies on Microfluidizer M-210 when compared to M-110, primarily due to the larger interactive chamber associated with the M-210.

In conclusion, a laboratory process can be scaled-up to a production batch on the Microfluidizer with ease for the studied formulation. Characteristics of liposomes that were produced on the production size unit (M210) compared fairly well with liposomes produced on the laboratory unit (M110).

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