Continuous production of liposomes and lipid nanoparticles using the 5 Input and Micromixer chip

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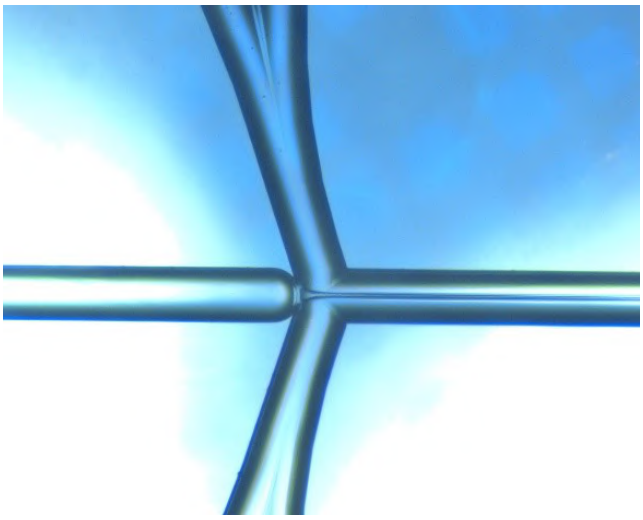

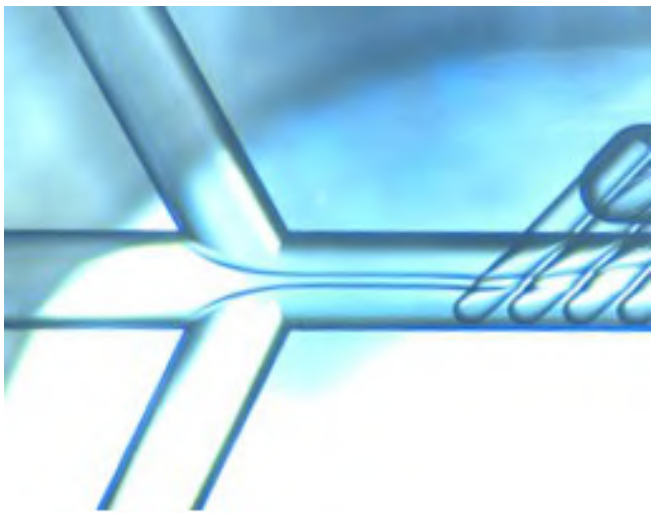

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Contents

1 Aims and Objectives

In this application note we demonstrate the use of the Dolomite System for the controllable production of liposomes and lipid nanoparticles (LNP) with two different chip geometries 1) hydro-dynamic flow focussing, 2) micromixing. Typical size range achieved using these chips is between 50 – 300 nm with total flow rates of 100 to 1000 µl/min.

2 Introduction

This application note provides all the information necessary to make liposomes using the Dolomite Liposome production system containing the 5 Input and Micromixer chip. Liposomes are spherical vesicles composed of one or more phospholipid bilayers. Lipid nanoparticles are like liposomes composed of phospholipids but different in composition and function. Lipid nanoparticles form a micelle-like structure, encapsulating Active Pharmaceutical Ingredients (API) in non-aqueous cores.

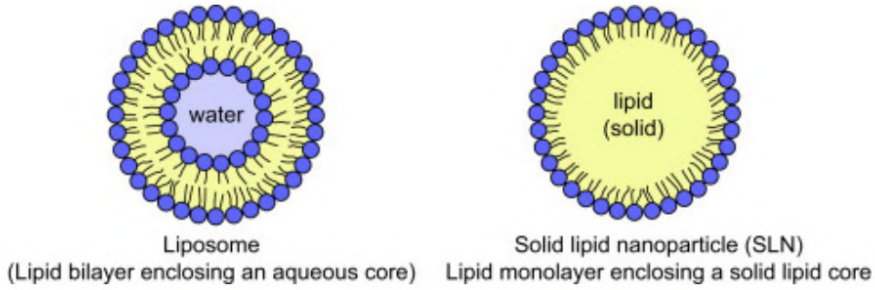


Figure 1 Illustration of liposomes and lipid nanoparticles. Adapted from Kammari et al 2017.

Liposomes have gained increasing attention from various industries due to their versatility in delivering both hydrophobic and hydrophilic drugs since they closely resemble the structure of cell membranes. Their natural composition and nano-size enable them to be effective carriers of active compounds to the human body reaching internal tissues and cells to deposit Active Pharmaceutical Ingredients (API). Thus, site-specific delivery with extended half-life is an attractive aspect for using liposomes in medicine. Many pharmaceutical active ingredients and biological compounds such as genetic materials, peptides, enzymes, chelating agents, vaccines, proteins, hormones, anticancer and antimicrobial agents have been encapsulated within liposomes particles and delivered by means of these vesicular structures [2].

However, there are several challenges that face the production and encapsulation in liposomes. Conventional methods (thin film hydration) lack the ability to precisely control liposome size and reproducibility. Polydisperse liposomes cannot easily be manipulated to control encapsulation efficiency and therefore prevents the API bioavailability. Doxorubicin (Doxil®), a chemotherapy drug delivered to cancer cells via liposomes was first commercialised in 1995 similarly Ambisome was commercialised to treat fungal infections. To date, little has changed in improving the production of liposome to better control size and encapsulation efficiency on a commercial scale.

In this application note we use microfluidics, (as demonstrated by various research articles) to address many of these concerns. Microfluidic platform allows for the precise real-time control over the production of liposomes which are not only reproducible but have a narrow size distribution. This automatically improves the encapsulation efficiency and therefore the efficacy of the API at the target site.

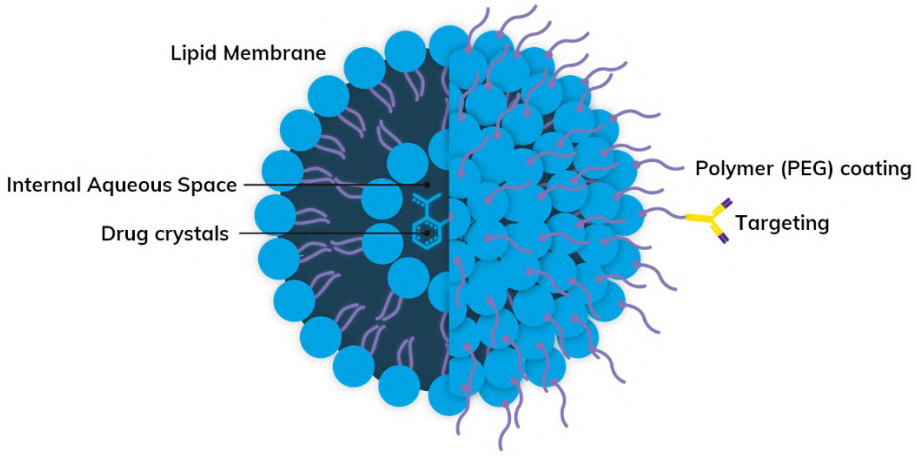


Figure 2 Liposome vesicle structure.

2.1 Microfluidic vs Batch Methods

Over the past few decades different techniques have been proposed for the preparation of liposomes. The process of liposome production defines its chemical purity and physical properties: morphology, size distribution, mean size, dispersity, lamellarity and entrapped liquid volume. Batch methods are widely employed in the synthesis of liposomes. However, liposomes typically manufactured in conventional batch stirred volumes are inadequate about process controllability and reproducibility of the final product. When working in batch systems, perfect mixing conditions are difficult to achieve. This leads to inhomogeneous distribution of concentration and temperature, which results in broad particle size distributions and substantial batch-to-batch product variability. Variations in particle characteristics are responsible for a wide range of formulation problems, related for instance to bioavailability.

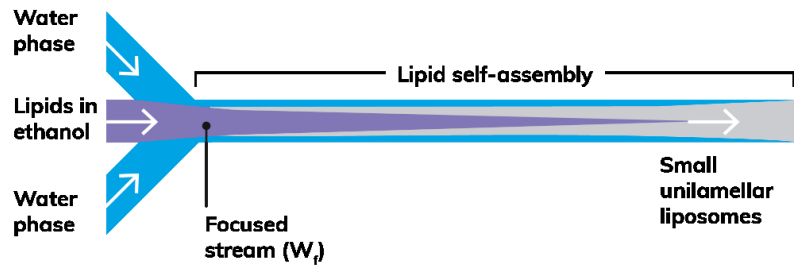
Recently, improvements have been made in development of microfluidic production methods for liposome synthesis. Microfluidic methods have demonstrated potential for achieving higher control over the physical properties of the final liposome product, particularly in terms of small mean size, narrow size distribution and lamellarity (Table 1). These characteristics make microfluidic approaches very attractive methods of liposome production for the pharmaceutical industry.

Dolomite Microfluidic	**Method Batch Method**
High encapsulation efficiency	Low Encapsulation efficiency
Monodisperse (CV < 5 %)	Polydisperse (CV > 20 %)
Scale to kg/day with no waste	~ 50% waste
Consistent from run to run	Poor batch to batch consistency
Uniform API distribution	Uneven API distribution
Precise particle size control	Poor particle size control
Wide range of particle size	Limited size range without sieving

Table 1 Microfluidic VS Batch Synthesis

2.2 Microfluidic hydrodynamic focusing (MHF)

The Microfluidic hydrodynamic focusing (MHF) technique developed by Jahn et al. in 2004 [3] presents the typical physical characteristics of microfluidic systems (i.e., including low Reynolds number and diffusion dominated mass transfer) and is the best viable microfluidic method for producing lipid-based nanoscale vesicular systems with potential for clinical application. The MHF method relies on the use of microfluidic devices with a cross flow geometry (Figure 3). Typically, a stream of lipid in alcohol solution is forced to flow in the central (or inner) channel of the device. The lipid stream is intersected and sheathed by two lateral (or coaxial) stream(s) of a water phase (typically distilled water or aqueous buffers). In this way, the lipid containing stream is hydrodynamically focused into a narrow sheet having a 3D cross-section preventing the inner stream contacting the wall and therefore preventing clogging. Notably, the size of the focused stream can be tuned by adjusting the volumetric flow rate ratio (FRR) between the water and lipid phase streams, and the total flow rate (TFR). The formation of liposomes in MHF chips is governed by the diffusion of different molecular species (mainly alcohol and water, but also lipids) at the liquid interface between the solvent (alcohol) and non-solvent (water) phases. The alcohol in which the lipids are initially solubilised diffuses into the water (and concomitantly the water diffuses into the alcohol) until the alcohol concentration decreases to a critical level, below the solubility limit of the lipids. As such, the alcohol diffusion triggers the formation of liposomes by a mechanism described as “self-assembly”. Specifically, it is believed that the reciprocal diffusion of alcohol and water across the focused alcohol/water interface causes the lipid to precipitate, resulting in the formation of intermediate structures, in the form of oblate micelles, that subsequently close upon themselves forming liposome vesicles [4].



TFR = total flow rate

$$\textbf{FRR} = \frac{\text{water phase flow rate}}{\text{ethanol flow rate}}$$

Figure 3 Schematic representation of an MHF geometry based on Jahn et al [3].

2.3 Micromixer chip

In the case of the Micromixer chip the laminar flow regime is replaced by 12 mixing stages. Here, liposomes precipitate during the mixing stages in a controlled and reproducible way. At each stage fluid mixing is increased resulting in a completely homogenous sample. Reproducibility is achieved by maintaining variables constant between runs such as concentrations, FRR and TFR. The mixing stages ensure that with each run a homogenous solution is produced with monodispersed liposomes hence why higher flow rates can be achieved.

3 Experimental method

3.1 Materials and methods

Firstly, lipid (Phospholipon 90G, Lipoid Switzerland) and Dimethyldioctadecylammonium (DDAB) (Fischer Scientific, UK) were dissolved in ethanol (reagent grade 99%, Sigma Aldrich, UK) at 10 $mgml^{-1}$ and 2.5 $mgml^{-1}$ respectively. All prepared solutions were filtered with a 0.2 µm filter prior to use. For the aqueous phase, phosphate buffer saline (PBS) at pH 7.4 was used. Both phases were loaded into the Dolomite pressure pumps (P-Pumps) in scintillation vials.

For both chips (5 Input 3D Chip (3200834) and Micromixer (3200401) the organic phase containing lipids and cholesterol flowed through the inner channel (dispersed phase) of the chip whilst the aqueous phase (PBS) flowed through the outer channels (continuous phase). Once a steady laminar flow was achieved, flow control was switched from pressure to flow mode. In flow mode, different flow rate ratios and total flow rates were investigated.

The size and the frequency of the liposomes was readily tuned by adjusting the flow rates of the dispersed and continuous phase. Upon collection, the samples were analysed using a Zetasizer (Malvern, UK) to determine the size, particle concentration and polydispersity index. The particle size distributions are a plot of the relative intensity of light scattered by the particles in various size classes and is therefore known as an intensity number distribution.

The experiments were repeated on separate occasions to show reproducibility. Once the process has been optimised, drugs or API can be incorporated into the liposomes for encapsulation testing.

3.2 System Setup

The system setup is shown in the Figure 4. The fluids are delivered using two Pressure Pumps (Part No. 3200016). The first Pump P1 delivers the Lipids/ethanol solution (yellow line) and works in combination with the Sensor Display (Part No. 3200095) and the 1-50 µl/min the Flow Rate Sensor (Part No. 3200098). The second Pump P2 delivers the PBS antisolvent solution (light blue line) and works in combination with the Sensor Display (Part No. 3200095) and the Flow Rate Sensor 30-1000 µl/min (Part No. 3200097). A third Pump may be required when encapsulating an API for the 5 Input chip setup since the API flows through the middle channels between the Lipid/ethanol and PBS channels.

Fluidic connections between the three P-Pumps and the two T-connectors (Part No. 3000397) are made using FEP tubing of OD 1.6 mm and ID 0.25 mm (Part No. 3200063). All the other connections are made using FEP tubing OD 1.6 mm and ID 0.25 mm (Part No. 3200302). 2-way in-line valves (Part No. 3200087) are placed on each fluid line to provide an easy-to-use solution to quickly stop flow streams. To ensure that fluids are equally divided using the T-connectors, the lengths of the tubes on each branch of the two T-connectors must be the same.

The fluids are delivered from the pumps to the 5 Input Chip (Part No. 3200834) or Micromixer chip (Part No. 3200401) as shown in (Figure 4). The 5 Input Chip is a hydrophilic flow focusing glass microfluidic device that allows the formation of a stable laminar stream confined by two lateral streams. The Micromixer is also a hydrophilic glass microfluidic device but allows mixing of fluids via its 12 herring bone mixing stages. They are both assembled with the H Interface (Part No. 3000155) and two Linear Connectors 4-way (Part No. 3000024).

Visualization is achieved using a High-Speed Digital Microscope (Part No. 3200531).

3.2.1 System Start-up and Shut down

Open valve V1 and start Pump P1 setting the desired flow rate of the antisolvent PBS solution. Then, open valve V2 and start pump P2. Increase gently the flow of the lipids/ethanol solution until a stable stream is created within the outlet channel. The thickness of the laminar lipids in ethanol stream can be controlled by changing the ratio FRR between the lipids in ethanol stream and the PBS stream. To shut down the system close first V2 and then V1. Finally, load Pump P1 and P2 with deionised water, open valve V1 and V2 and start the pumps to flush the system and clean the channel surfaces from any particles deposited.

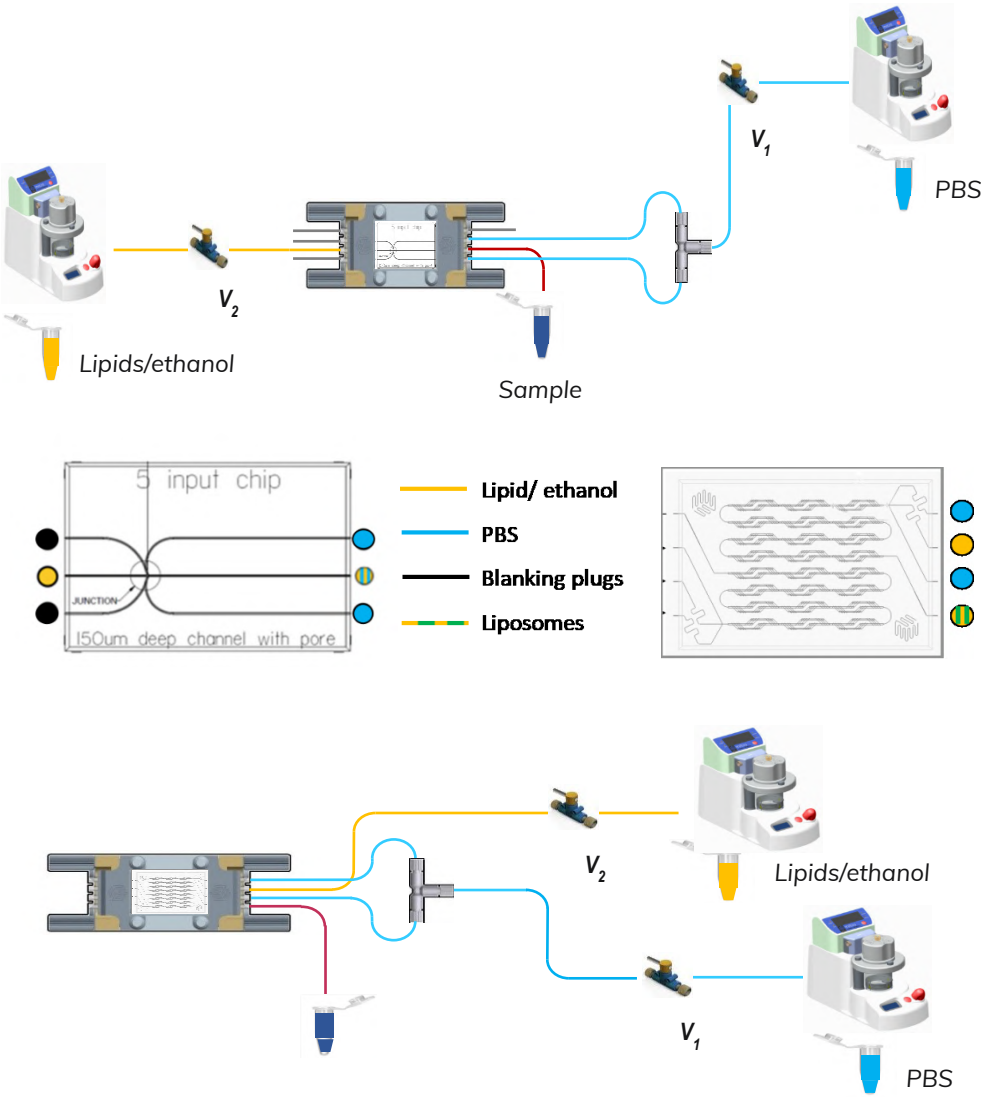


Figure 4 Schematic showing representative setup of a nanoliposome system using (Top) 5 Input chip (Bottom) Micromixer chip.

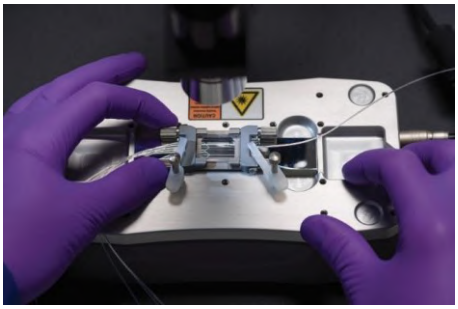
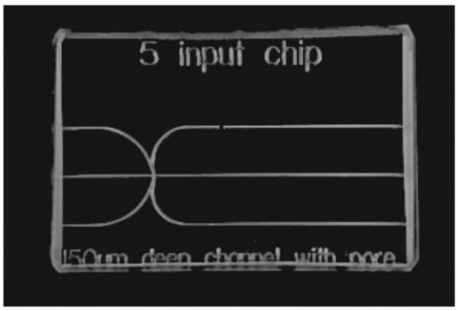


Figure 5 Liposome Synthesis System



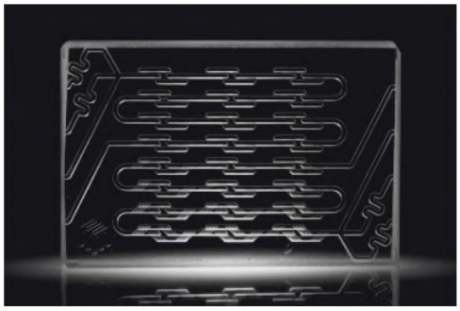


Figure 6 5 Input chip and Micromixer chip for liposome synthesis

The effect of the flow ratio FRR on the liposome particles size is investigated using the flows and flow ratios FRR reported in Table 2. According to the literature the size of liposome is mainly affected by FRR. The total flow rate was investigated mainly for the Micromixer chip due to its capability in handling higher flow rates. A total flow rate (TFR) rounded to the nearest hundred, equal to 100 µl/min is fixed in all the experiments for the 5 Input chip while changing FRR. For the Micromixer chip total flow rate was varied from 100 µl/min, 500 µl/min and 1000 µl/min (to the nearest five hundred) whilst changing the FRR. Subsequent graphs referring to TFR of 100, 500 and 1000 µl/min show an approximate value of the actual TFR found in Table 2-5, this was done because lipid/ethanol was calibrated using the flow sensor against the selected fluid "water" in FCC. The calibration can be found in Figure 14 (appendix) which was used to give the actual values of the flow rate of lipids/ethanol.

$F_{lipids/EtOH}$ µl/min (value entered on FCC)	F_{PBS} µl/min	TFR = $F_{lipids/EtOH}$ + F_{PBS} µl/min	FRR = F_{PBS} / $F_{lipids/EtOH}$ µl/min
59 (20)	80.0	139	1
17 (5.8)	94.2	111	5
14 (4.8)	95.2	109	7
7 (2.5)	97.5	105	13
6 (2.1)	97.9	104	16
4 (1.2)	98.8	102	28
3 (0.87)	99.1	102	38
2 (0.7)	99.3	101	48
1 (0.5)	99.5	101	67

Table 2 FRR used with the 5 Input chip at TFR ~100 µl/min.

$F_{lipids/EtOH}$ µl/min (value entered on FCC)	F_{PBS} µl/min	TFR = $F_{lipids/EtOH}$ + F_{PBS} µl/min	FRR = F_{PBS} / $F_{lipids/EtOH}$ µl/min
59 (20)	80	139	1
17 (5.8)	94.2	111	5
14 (4.8)	95.5	109	7
7 (2.5)	97.5	105	13
6 (2.1)	97.9	104	16
4 (1.2)	98.8	102	28
3 (0.87)	99.1	102	38
2 (0.7)	99.3	101	48
1 (0.5)	99.5	101	67

Table 3 FRR used with the Micromixer chip at TFR ~100 µl/min

$F_{lipids/EtOH}$ µl/min (value entered on FCC)	F_{PBS} µl/min	TFR = $F_{lipids/EtOH}$ + F_{PBS} µl/min	FRR = F_{PBS} / $F_{lipids/EtOH}$ µl/min
296 (100.0)	400.0	696	1
89 (30.0)	470.0	559	5
65 (22.0)	478.0	543	7
37 (12.5)	487.5	525	13
31 (10.5)	489.5	521	16
17 (5.9)	494.1	512	28
13 (4.4)	495.6	509	38
10 (3.5)	496.5	507	48
7 (2.5)	497.5	505	67

Table 4 FRR used with the Micromixer chip at TFR ~500 µl/min

$F_{lipids/EtOH}$ µl/min (value entered on FCC)	F_{PBS} µl/min	TFR = $F_{lipids/EtOH}$ + F_{PBS} µl/min	FRR = F_{PBS} / $F_{lipids/EtOH}$ µl/min
592 (200.0)	800.0	1392	1
178 (60.0)	940.0	1118	5
118 (40.0)	960.0	1078	7
74 (25.0)	975.0	1049	13
62 (21.0)	979.0	1041	16
36 (12.0)	988.0	1024	28
26 (8.7)	991.3	1017	38
21 (7.0)	993.0	1014	48
15 (5.0)	995.0	1010	67

Table 5 FRR used with the Micromixer chip at TFR ~1000 µl/min

4 Results

4.1 5 Input Chip

Experiments were conducted using the 5 Input chip as per the parameters highlighted in Table 2. Figure 7 shows the relationship between the FRR and the liposome size for the same TFR of 100 µl/min. The relative error bars in Figure 7 show the standard deviation (SD) calculated for the set of three experiments for each FRR. DLS particle concentration as a function of size for three different FRR is plotted in Figure 10. These concentrations are determined by the DLS instrument assuming spherically shaped particles.

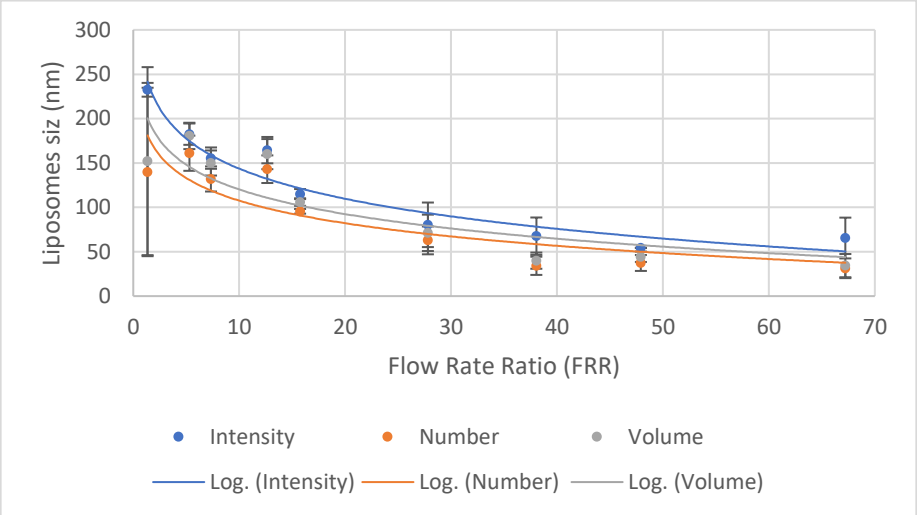


Figure 7 Peak intensity, number and volume of liposome nanoparticles at different FRR, TFR 100 µl/min produced using the 5 Input chip. Average of 3 set of measurements.

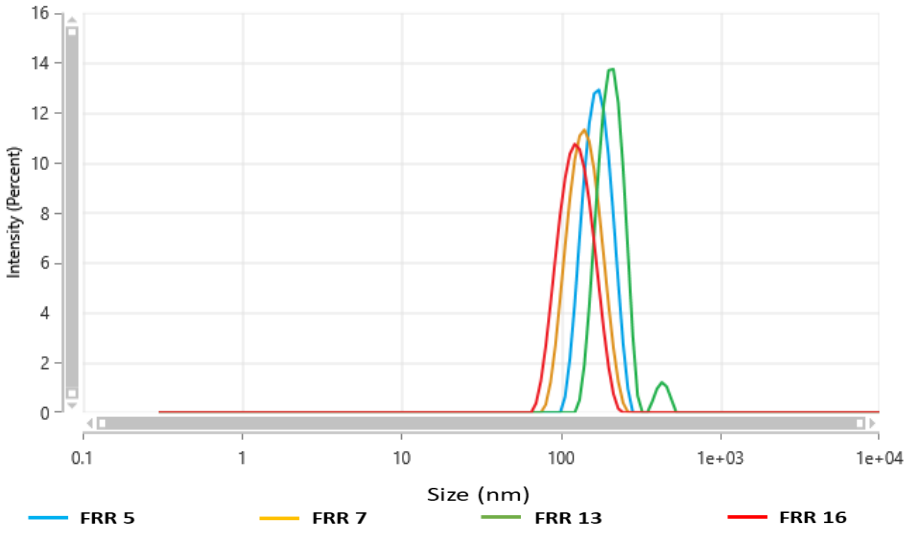


Figure 8 Peak intensity, number and volume of liposome nanoparticles at different FRR 5,7,13,16 with TFR 100 µl/min produced using the 5 Input chip.

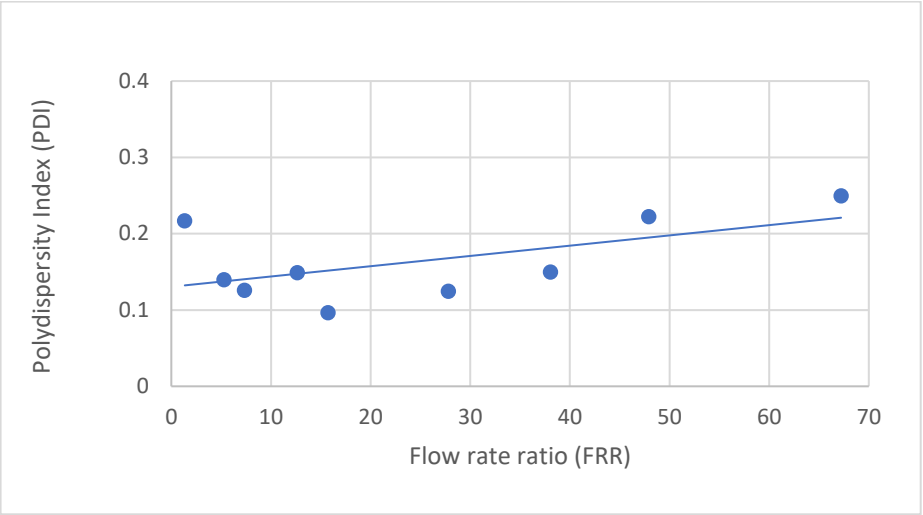


Figure 9 Polydispersity index for the tested FRR (table 2) at TFR 100 µl/min produced using the 5 Input chip.

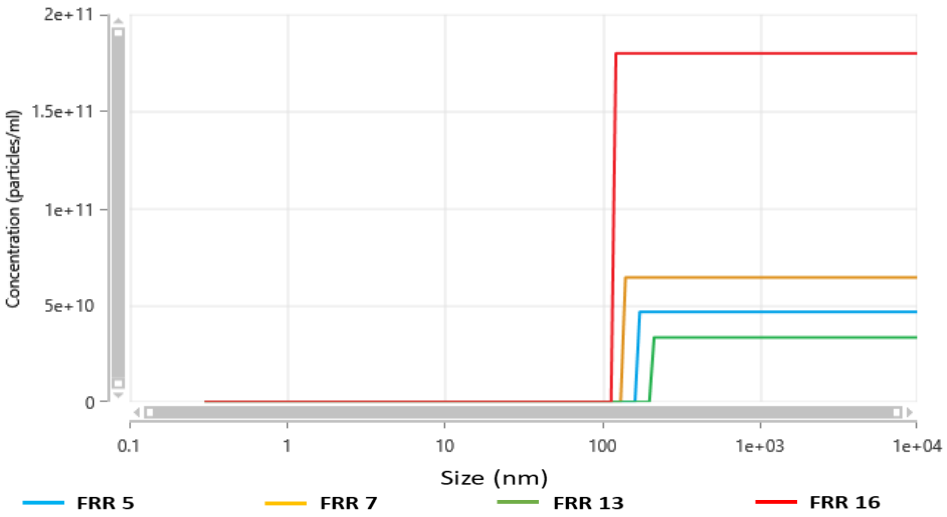


Figure 10 Particle concentration of liposome nanoparticles at different FRR (5,7,13,16) with TFR 100 µl/min produced using the 5 Input chip.

4.2 Micromixer Chip

Experiments reported here were performed using the Micromixer chip as per the parameters highlighted in Table 3-5. Three sets of experiments were carried out at TFR 100, 500 and 1000 µl/min, whilst changing the FRR. Figure 11 shows the liposome size analysis data obtained from DLS demonstrating the change in average liposome size as a function of FRR. The relative error bars in Figure 11 show the standard deviation (SD) calculated for the set of three experiments for each FRR. The DLS intensity, number and volume graph are also reported showing the shift in peaks with changing FRR in Figures 12. DLS particle concentration as a function of size for four different FRR is plotted in Figure 13. These concentrations are determined by the DLS instrument assuming spherically shaped particles.

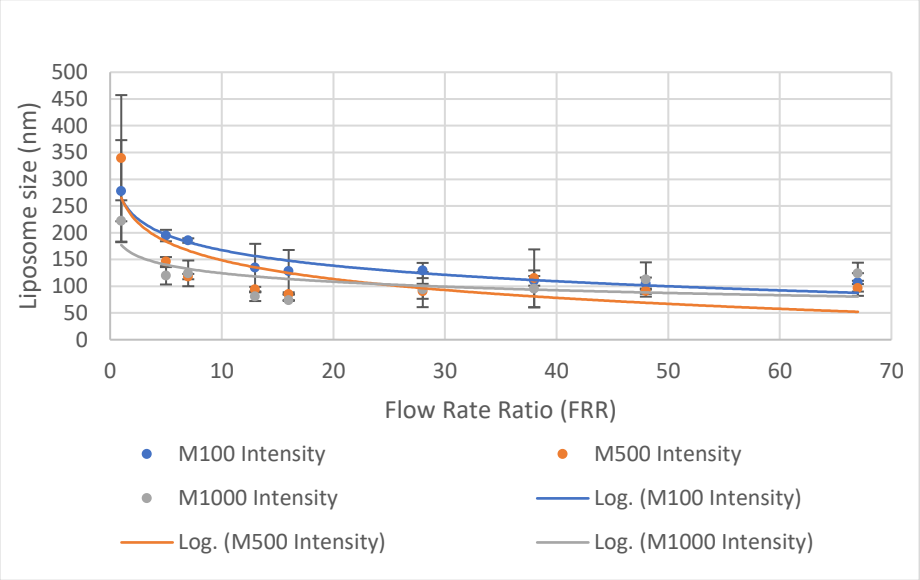


Figure 11 Peak intensity of liposome nanoparticles produced using a Micromixer chip at different FRR with TFR of 100 (M100), 500 (M500 and 1000 (M1000) µl/min. Average of 3 set of measurements.

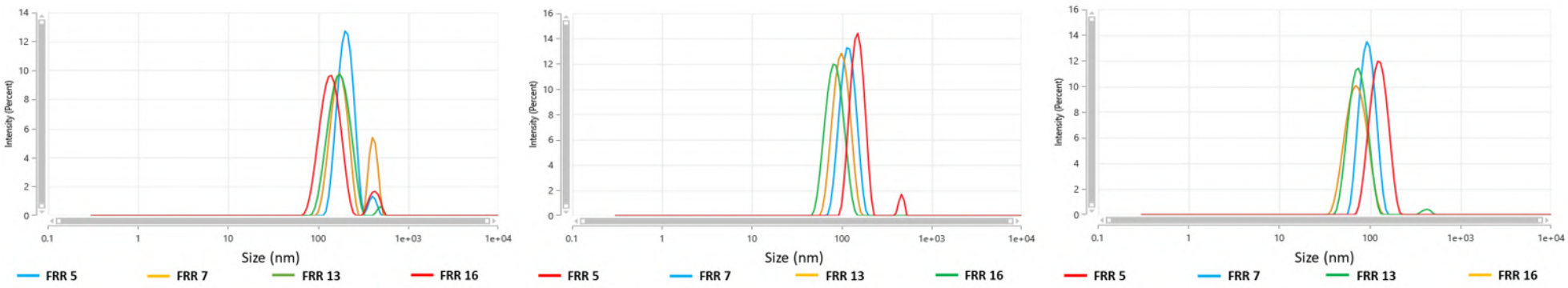


Figure 12 Peak intensity of liposome nanoparticles at different FRR for TFR a) 100 µl/min, b) 500 µl/min and c) 1000 µl/min using the Micromixer chip

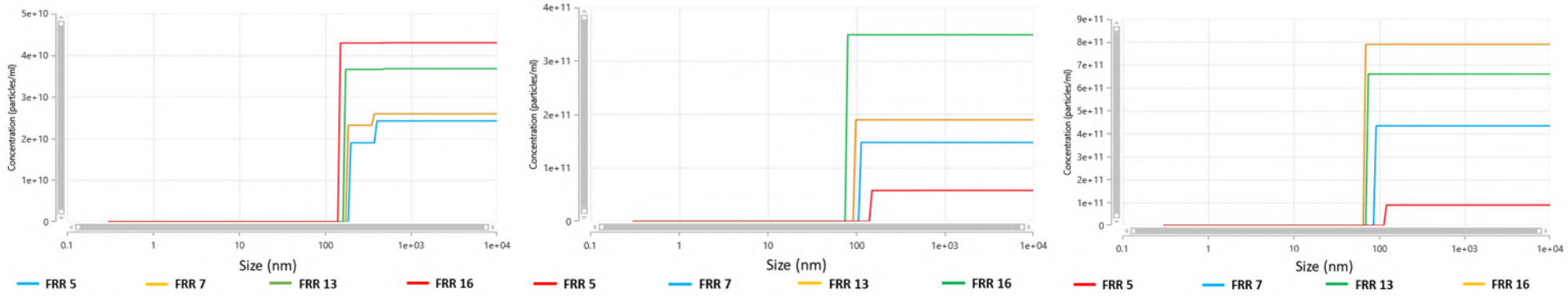


Figure 13 Particle concentration of liposome nanoparticles at different FRR for TFR a) 100, b) 500 and c) 1000 µl/min using the Micromixer chip.

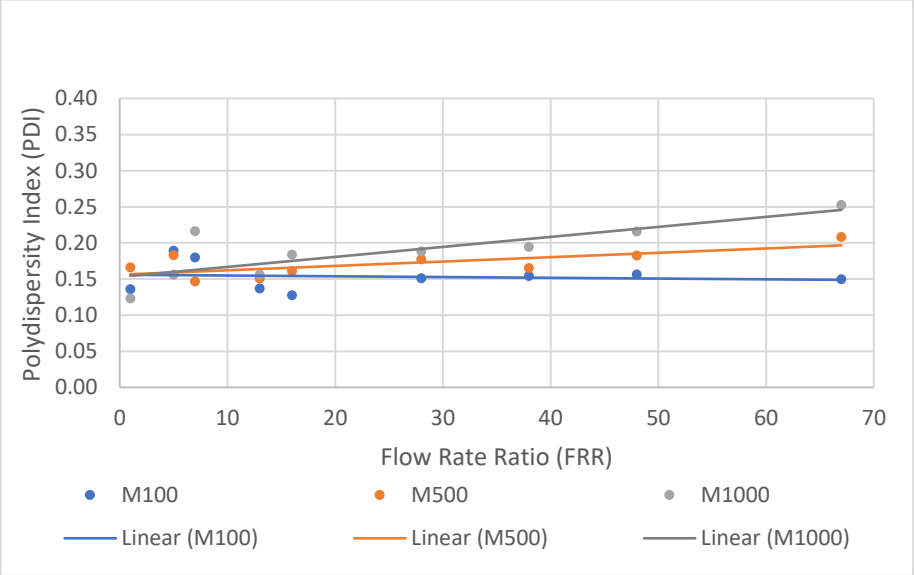


Figure 14 PDI of liposomes produced with the Micromixer chip at various FRR and TFR.

5 Discussion

The results show that liposomes are produced approximately in the range between ~ 50 to 230 nm for the 5 Input chip and ~ 50 to 300 nm for the Micromixer chip based on intensity distribution. Particle mean size can be tuned by controlling the flow ratio FRR. An increase of the flow ratio FRR leads to a decrease of the nanoparticle size with precise control that cannot be achieved with traditional batch synthesis.

As reported in the previous sections, this finding agrees with the literature of liposome formation using MHF methods [5-10]. The several MHF profiles achieved by mixing ethanol/lipids and PBS under different FRR result in laminar focused streams of different width in the 5 Input chip. This condition allows for mixing based entirely on molecular diffusion in a direction normal to liquid flow streamlines. At a critical water-to-alcohol concentration the lipid monomers in the alcohol stream exceeds the solubility level and spontaneously self-assemble in a supersaturated environment into spherical structures that enclose the surrounding fluid (vesicles).

The effect of both TFR and FRR parameters has been recently investigated. Jahn et al. first found that the mean size of the liposome produced is inversely proportional to FRR and directly related to lipid concentration. Conversely, a small effect on liposome size was observed when TFR is changed [5 - 6]. These results were confirmed in subsequent studies such as Hood et al. in 2013, Balbino et al. in 2013 and Mijajlovic et al. in 2013 [7 - 9]. Recently, Carugo et al. in 2016 carried out an important parametric study showing for the first time the higher properties of nanoliposomes particles produced by MHF. The author demonstrated the potential of MHF systems showing for the first time that liposomes generated by this technology are smaller and more uniform, size can be tuned by adjusting the flow rate ratio FRR and these vesicles have a higher encapsulation efficiency than the ones synthesized by traditional batch methods [10].

Figure 7 and 11 show the average size distribution of each FRR tested for both chips. There is a clear correlation between the FRR and the average liposome size in all respects. As the FRR is increased the liposome size decreases because the concentration of lipid for a given volume of PBS decreases. This enables rapid diffusion and precipitation of liposomes. At low FRR i.e. FRR 1 the concentration of lipids per a given volume of PBS is increased therefore the time required for complete assembly of liposomes is increased resulting in bigger liposomes. Also, since the concentration is higher the likelihood of aggregates (see Figure 16 in appendix) also increases. The presence of aggregates means the liposomes may be polydisperse and not reproducible.

Figures 7 and 11 demonstrate the shift in liposome size in relation to the FRR. The peak intensities move to the left as the liposome size gets smaller. Not all FRR are shown in these graphs due to over-crowding, the graphs can be obtained via request (applications@dolomite-microfluidics.com). Polydispersity index is shown by Figure 9 and 14, in both chips the polydispersity index overall is below 0.25 for all parameters tested. In certain cases, the PDI is 0.1 meaning the liposomes are extremely monodispersed and highlight the optimised parameters for the formulation.

In addition to the size analysis, particle concentration was also examined, Figure 10 and 13. The figures demonstrated the particle concentration increases as the FRR increases. Smaller liposomes produced at high FRR are detected at a higher frequency than bigger ones resulting in high particle concentration for high FRR.

6 Conclusions

This application note demonstrated the synthesis of liposome nanoparticles using Dolomite's nanoliposome generation system.

Liposome nano vesicles traditionally synthesized by bulk methods are generally not a uniform and reproducible. Consequently, these particles do not represent a very attractive solution for pharmaceutical industry; particularly in the field of smart drug delivery where narrow distributions, small particle sizes and controllable and reproducible synthesis are desired. Nowadays, new microfluidic continuous flow technologies offer an attractive solution for the synthesis of small and narrow size particle distributions. Decreasing the sample stream width to micro-meter length scales allows for controlled and reproducible mechanical and chemical conditions across the stream width. This results in more utilization of materials as none of the product size falls outside the allowable size limits for in-vivo use.

In this work liposome nanoparticles in the range between 50 nm and 300 nm were produced using the microfluidic hydrodynamic focusing (MHF) and mixing strategy. The benefit of using the Micromixer chip is that it gives a wider size range with higher throughput. The 5 Input chip enables better control over the polydispersity index.

Particle size can be controlled by changing the ratio of the phases mixed. The ability to synthesise liposome nanoparticles in a more controllable and reproducible way opens possibilities for custom tuning of surface properties. This is achievable by adding surfactants or API to the lipid mix, or by adding downstream processes. As the entire chemistry is user controlled, Dolomite's liposome generation system enables users to manipulate the entire synthetic route in-house with control of purity standards.

Scale-up from the R&D system is also available via multiplexing the channels on a single chip. Our Telos Micromixer chip (3200832) is currently available for scale-up process. Each chip contains seven channels increasing throughput not only seven times, but the chips can be stacked up to ten times enabling system scale up of seventy times. The subject of future application notes will be high throughput.

With the rapid development of microfluidic manipulation methods, new nanoparticle synthetic methods with better control and design of nanoparticle properties are expected in the coming years.

7 Appendix

7.1 Calibration Curve

Calibration curves allow to calculate the actual flow rates of non-standard fluids from the set flow rate of common standard fluids. The calibration chart reported below is used to determine the actual lipid and cholesterol flow rates at reference (set flow rate) read by the flow sensor which is set on water as working fluid on FCC.

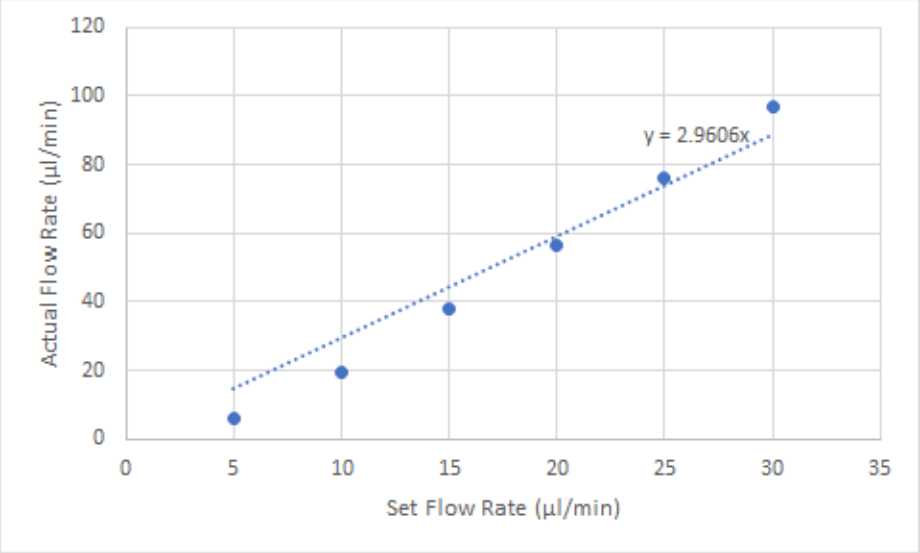


Figure 15 Calibration curve for various concentrations of lipid and cholesterol in ethanol

Micromixer chip can attain high throughput in comparison to the 5 Input chip because of the difference in chip geometry. The residence time in the outlet of the 5 Input chip is shorter therefore depending on the formulation, higher TFR will result in liposome assembly downstream of the collection channel. In such situations the formulation has an important role in deciding the outcome of the liposome monodispersity. Micromixer chip does not rely on fluid diffusion alone instead it uses active mixing to assemble liposomes meaning higher TFR can easily be achieved without modifying other variables.

DLS size analysis measures the correlation function of intensity fluctuations but also reports number and volume based on mathematical models incorporated in the software. Both number and volume are derived from intensity measurements. All three represent the data in different ways therefore by reporting only one can often mislead or skew the results. In this application note we report the data in all three formats to allow users to gain better visibility (number and volume data in appendix). For example, when reporting number distribution, the model usually presents the data as a single peak which may not necessarily be the case when looking at intensity distribution that is derived from the raw signal.

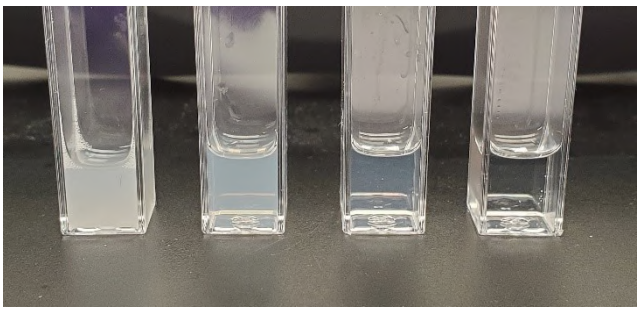


Figure 16 Cuvettes showing liposome aggregation. High (left) to no (right) aggregation.

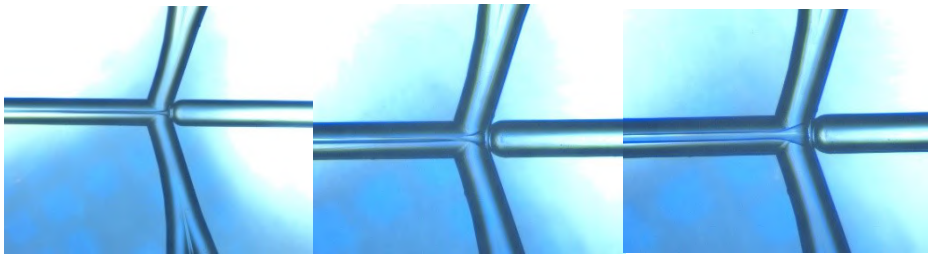


Figure 17 MHF streams at different FRR.

7.2 System Component List

Part No.	Description	Quantity
3200679	Liposome generation system – nanoliposomes – Enhanced Control The system includes:	1
	Mitos P-Pumps	3
	Sensor Displays	3
	Flow Rate Sensors	3
	High-Speed Digital Microscope	1
	Valves, Chip Interfaces, Fittings and Tubing	-
	Mitos Compressor 6bar	1
3200834	5 Input Chip - 150µm - Hydrophilic	3
3200401	Micromixer Chip	3
	Installation and Training	-

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