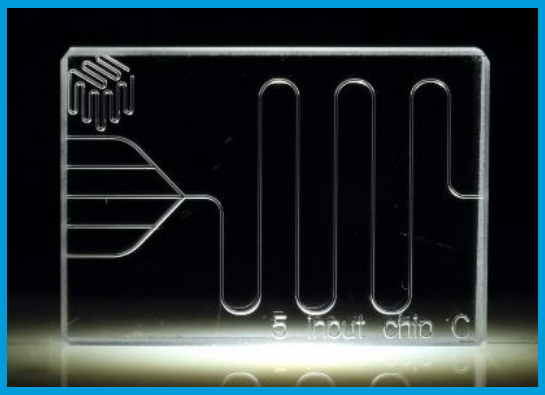
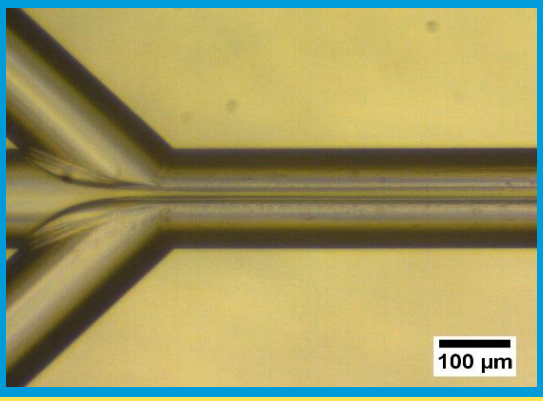
Continuous Microfluidic Synthesis of Liposome Nanoparticles by Hydrodynamic Flow Focusing

Dolomite's Nanoliposome Generation System





dolomite

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Aim & Objectives

In this application note we demonstrate the use of the Dolomite System for the controllable production of liposome nano-vesicles with final particle size in the range typically between 20 nm and 500 nm. The system is based on continuous microfluidic synthesis of liposomes using hydrodynamic flow focusing techniques by means of the Dolomite's 5 Input Chip - Long Channel.

Introduction

Liposomes are vesicular nano and micro-particles formed by one or more lipid bilayer membranes that encapsulate an aqueous volume. The lipid bilayer is constituted by amphiphilic molecules (*phospholipids*) (Figure 1). These molecules generally consist of two hydrophobic fatty acid "tails" and a hydrophilic phosphate group "head". Hydrophilic drugs can be loaded into the interior aqueous core of liposomes, whereas lipophilic and amphiphilic drugs can be incorporated into the lipid bilayers. The liposome lipid bilayer mimics the cell membrane lipid bilayer, thus delivering the liposome contents within the body. Special ligands can be attached to liposome surface allowing for their accumulation in pathological areas for treatment of disease. Control of liposome size is a crucial characteristic, as only vesicles of a precise size range can target a specific organ or disease. Once liposomes reach a specific site they are digested by natural macrophage phagocytosis, thus releasing its drug [1]. This makes liposomes very attractive biological systems that are widely employed as *drug delivery vehicles* as part of an expanding market. 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].

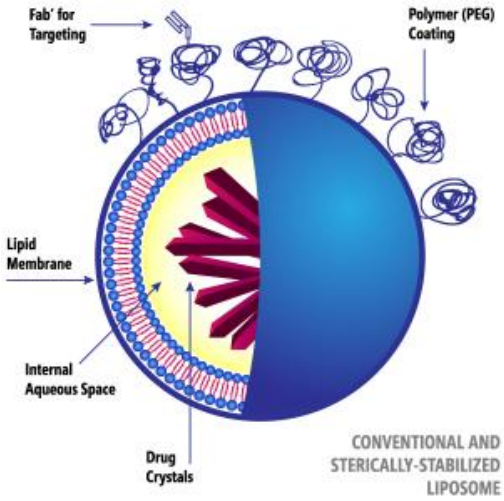

Figure 1. Liposome vesicle.

Microfluidic Synthetic Method of Liposome Nanoparticles

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 with regard to 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
~100% encapsulation efficiency	~30% 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 size control
Wide range of particle size	Limited size range without filtering

Table 1. Microfluidic VS Batch Synthesis.

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 2). 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 rectangular cross-section. 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].

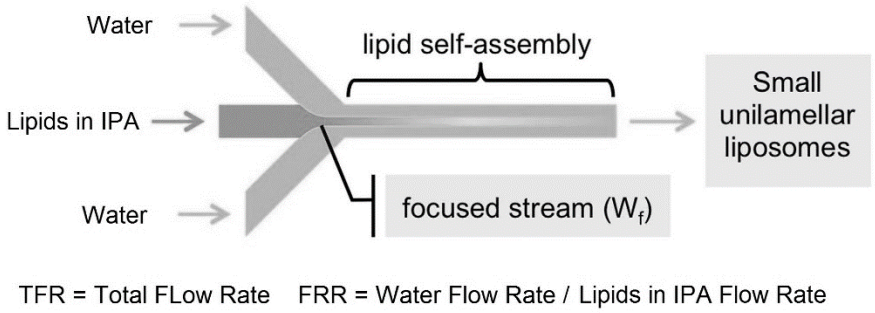

Figure 2. Schematic representation of a MHF geometry based on Jahn et al [3].

The effect of both TFR and FFR 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].

Experimental Setup

System Description

The system setup is shown in the Figure 3. The fluids are delivered using three Pressure Pumps (Part No. 3200016). The first Pump P_1 delivers the isopropyl alcohol (IPA) flushing fluid (red line) and works in combination with the Sensor Display (Part No. 3200095) and the Flow Rate Sensor 30-1000 µl/min (Part No. 3200097). The second Pump P_2 delivers the Lipids/IPA 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 third Pump P_3 delivers the Water 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).

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 0.8 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. 3200712) as shown in (Figure 3Figure 5**Error! Reference source not found.Error! Reference source not found.**). 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 chip has a long channel serpentine of 82 mm (Figure 5). It is assembled with the H Interface 7-way (Part No. 3200297) and two Linear Connectors 7-way (Part No. 3200148).

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

System Start-up and Shut-down

Open valve V_3 and start Pump P_3 setting the desired flow rate of the antisolvent Water solution. Then, open valve V_2 and start pump P_2. Increase gently the flow of the lipids/IPA solution until a stable stream is created within the outlet channel. During this stage keep the Pump P_1 and the valve V_1 closed. The thickness of the laminar lipids in IPA stream can be controlled by changing the ratio FRR between the lipids in IPA stream and the Water stream. To shut down the system close first V_2 and then V_3. Finally open the valve V_1 and start the pump P_1 to flush the system and clean the channel surfaces from any particles deposited.

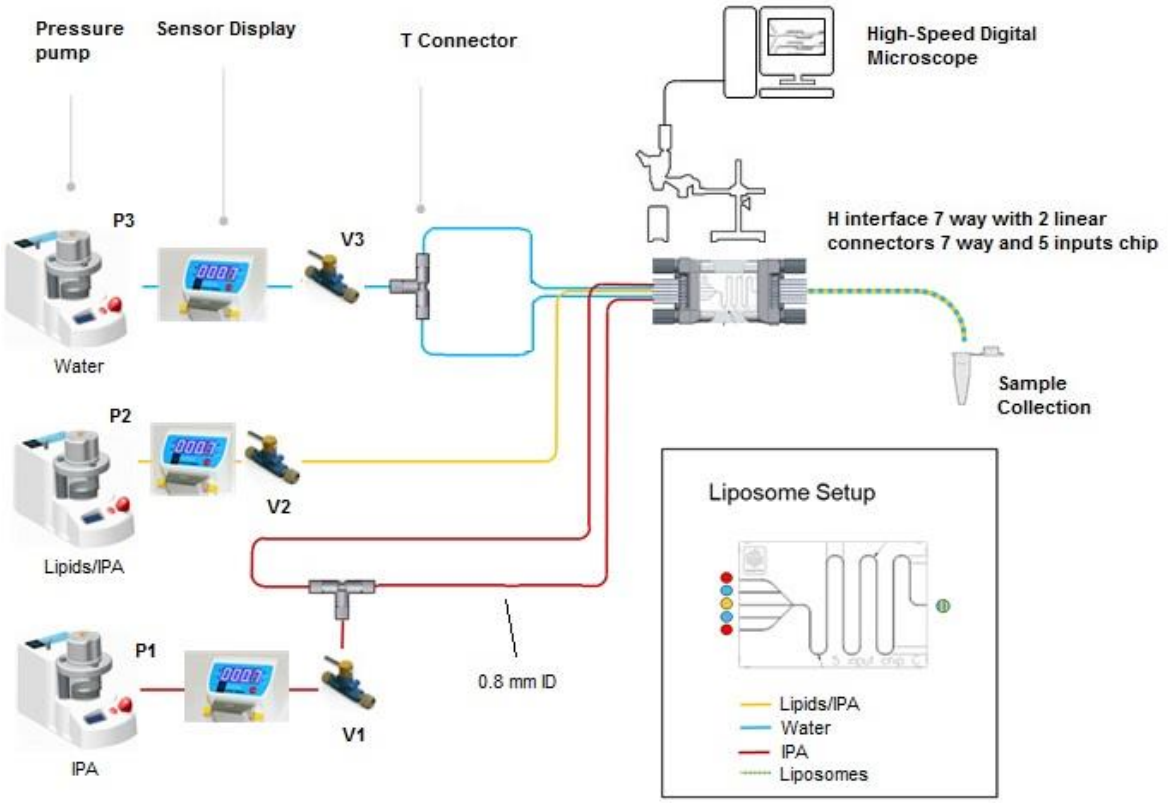

Figure 3. Schematic showing representative setup of a nanoliposome system.

Figure 4. Nanoliposome system.

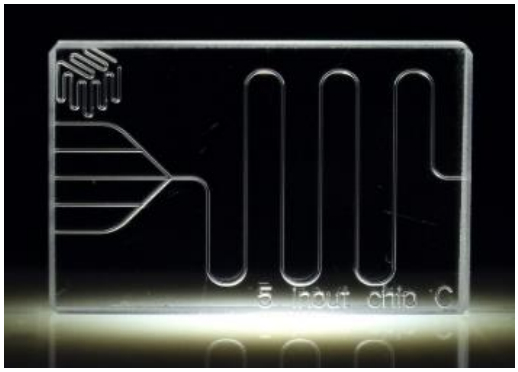

Figure 5. 5 Input Chip - Long Channel.

The lipids mixture is composed of Lecithin 90% soybean (LC), Cholesterol 95% stabilized (CH) and Dimethyldioctadecylammonium (DDAB) all purchased from Fisher Scientific. The DDAB is a double-chained quaternary ammonium surfactant that helps the formation of unilamellar vesicles in water. A mixture of 1g LC : 0.1g CH : 0.1g DDAB is suspended in 10ml of isopropyl alcohol at room temperature and stirred for 8h at T = 25ºC. The saturated solution is then collected and filtered by means of a Millipore 0.2 µm filter. Deionized water is used as antisolvent.

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. Therefore, the effect of the total flow rate (TFR) employed and the resulting residence time are not a subject of primary investigation. A total flow rate equal to 150 µl/min is fixed in all the experiments while changing FRR.

$F_{Lipids/IPA}$ $[\mu l/min]$	F_{Water} $[\mu l/min]$	$TFR = F_{Water} + F_{Lipids/IPA}$ $[\mu l/min]$	$FRR = F_{Water}/F_{Lipids/IPA}$ $[-]$
10	140	150	14
12.5	137.5	150	11
20	130	150	6.5
40	110	150	2.75

Table 2. Flows and FRR used with the 5 Input Chip - Long Channel.

Each experiment is conducted three time and particle number distributions and peak intensities are determined by Dynamic Light Scattering (DLS). The particle distributions obtained are a plot of the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity number distribution. Particles are collected at the outlet and analysed with DLS without further dilution.

Results & Discussion

Effect of FRR on liposome number distribution

Figure 6 reports the DLS number distributions obtained at different FRR for the same TFR = 150 µl/min. The corresponding peak intensities are plotted in Figure 7 with relative error bars showing the standard deviations (SD) calculated for the set of three experiments for each FRR. DLS particle concentration as a function of size for different FRR is plotted in Figure 8. These concentrations are determined by the DLS instrument assuming spherically shaped particles.

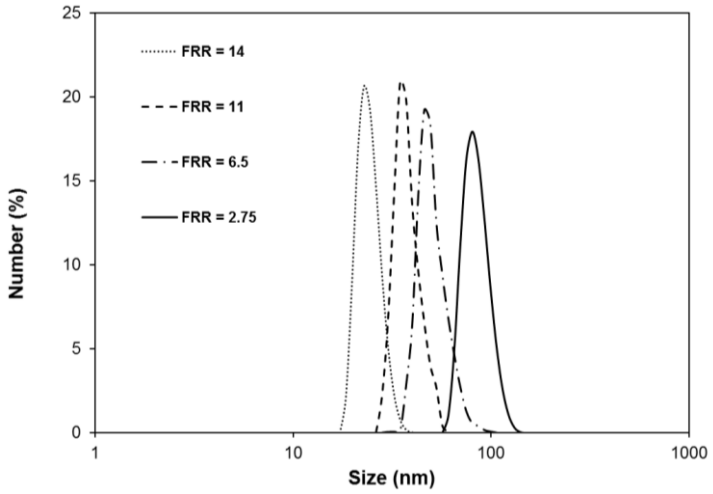

Figure 6. Number distributions of liposome nanoparticles at different FRR. TFR = 150 µl/min. Average of 3 set of measurements.

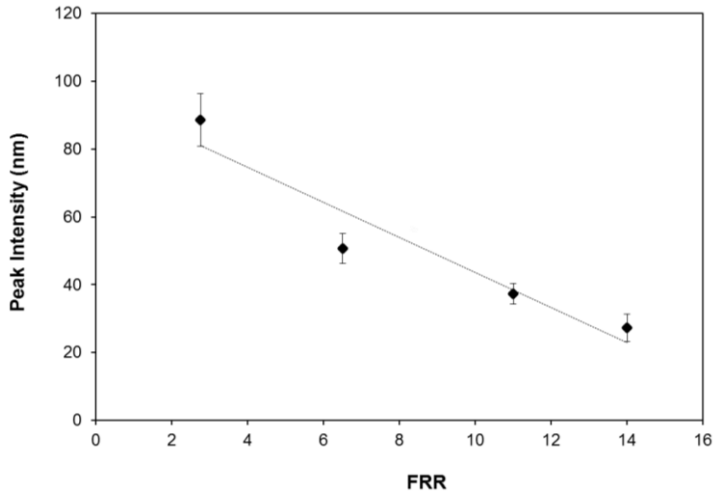

Figure 7. Peak intensities of liposome nanoparticles at different FRR. TFR = 150 µl/min. Average of 3 set of measurements.

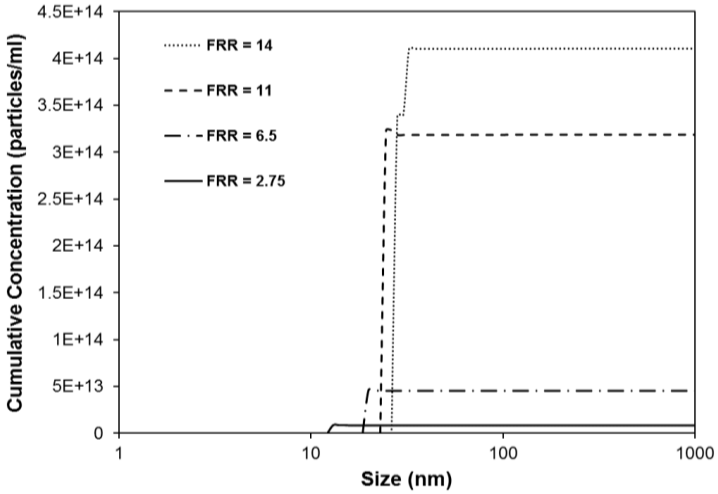

Figure 8. Cumulative particle concentration of liposome nanoparticles at different FRR. TFR = 150 µl/min. Average of 3 set of measurements.

The results show that liposome nanoparticles are produced in the range between ~ 30 nm and ~ 90 nm. Particle mean size (peak intensity) 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 a precise control that cannot be achieved with traditional batch synthesis. As reported in the previous sections, this finding is in agreement with the literature of liposome formation using MHF methods [5]–[10]. The several MHF profiles achieved by mixing IPA/lipids and water under different FRR result in laminar focused streams of different width (Figure 9). 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). Smaller widths of the MHF (high FRRs) result in reduced diffusion lengths for

mixing, thereby reducing the distance to reach the critical alcohol concentration where lipids spontaneously self-assemble into spherical vesicles. In these conditions of high supersaturation gradients, nucleation rates increase exponentially giving the production of many particles per unit volume of small size (Figure 7 and Figure 8). At the lowest FRR = 2.75 we produce $8.5 \cdot 10^{12}$ particles/ml of about 90 nm, as compared to FRR = 14 which gives $4.1 \cdot 10^{14}$ particles/ml of about 30 nm. This is qualitatively evident by looking at the transparency of the final solutions collected in vials prior to DLS analysis (Figure 10).

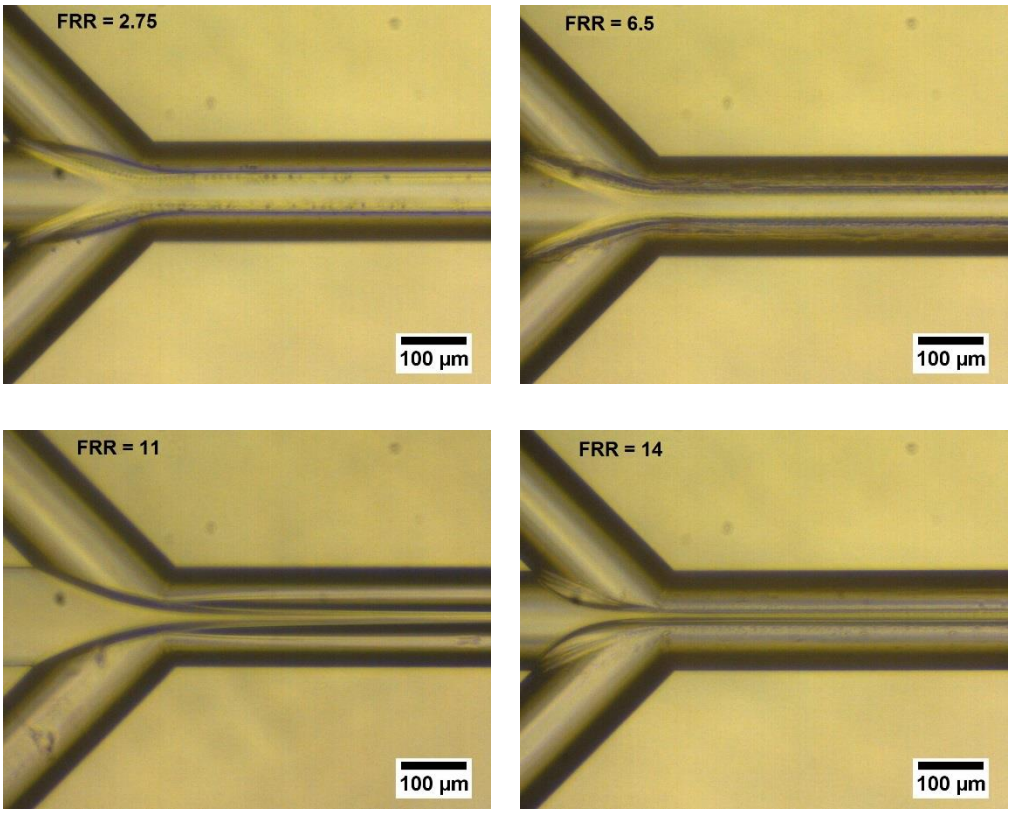

Figure 9. MHF streams at different FRR. TFR = 150 µl/min. Liposome particles precipitating at the interface between the two phases.

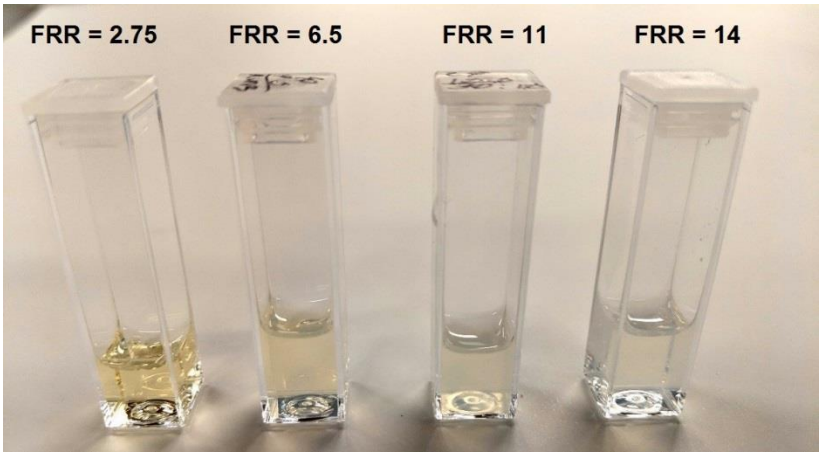

Figure 10. Liposome particles produced at different FRR. TFR = 150 µl/min.

The MHF streams generated at the beginning of the 5 Input Chip remain stable and symmetrically focussed along the channel serpentine as showed in the pictures below. This condition ensures reproducible fluid mixing and particle precipitation within the entire channel outlet.

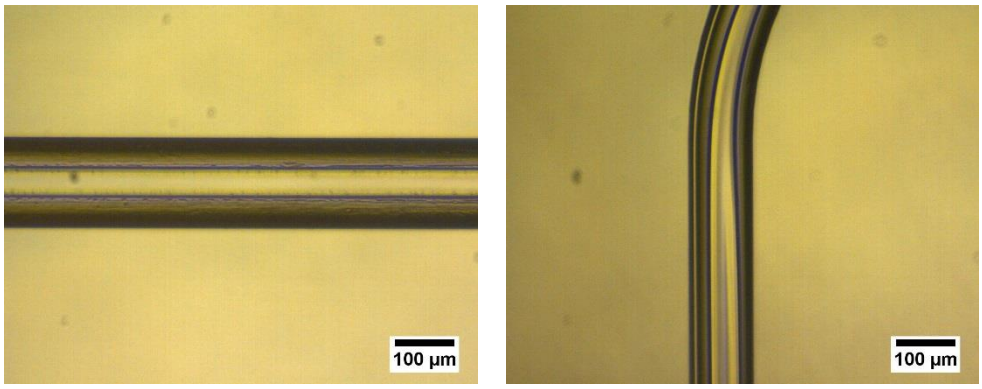

Figure 11. MHF stream for FRR = 6.5. TFR = 150 µl/min. Liposome particles precipitating at the interface between the two phases.

For the same batch of lipid mixture, the peak intensity of the several number distributions is typically reproducible within a SD = 8 - 15 nm between different experiments. Nanoliposome distributions are stable over 2 weeks within about 20 nm of the peak intensity measured by DLS. This demonstrates the robustness of on-chip synthesis for the selected lipid mixture.

Liposome production rate can be scaled up using the Dolomite Telos System for high throughput. This system offers the possibility to work with up to 70 MHF junctions arranged in a parallel configuration and working simultaneously. The possibility of high-throughput microfluidic architectures to reach mass production volumes has been recently presented and investigated experimentally by Carugo et al. (2016) [10].

The Nanoliposome Generation System presented in this application note is provided with an additional 5 Inputs Chip of shorter channel outlet $L = 16\ mm$ (Part No. 3200711) which allows users to work with shorter residence times and optimise and play with vesicle self-assemble mechanism.

Conclusions

In this application note we demonstrated the synthesis of liposome nanoparticle using Dolomite's nanoliposome generation system.

Liposome nano vesicles traditionally synthesized by bulk methods are generally not a uniform and reproducible product. 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 30 nm and 90 nm were produced using the microfluidic hydrodynamic focusing (MHF) strategy. 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 up 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.

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.

Appendix: System Component List

Part No.	Part Description	#
3200679	Liposome generation system - nano-liposomes - Enhanced Control The system includes:	-
	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
3200711	5 Input Chip (short channel), hydrophilic	3
3200712	5 Input Chip (long channel), hydrophilic	3
	Installation and Training	-

Bibliography

[1] V. Torchilin, “Multifunctional nanocarriers,” *Adv Drug Deliv Rev.*, vol. 58, no. 14, pp. 1532–55, 2006.

[2] Q. Fan, Y. & Zhang, “Development of liposomal formulations: From concept to clinical investigations,” *Asian J. Pharm. Sci.*, vol. 90, pp. 8–79, 2013.

[3] A. Jahn, W. N. Vreeland, M. Gaitan, and L. E. Locascio, “Controlled Vesicle Self-Assembly in Microfluidic Channels with Hydrodynamic Focusing,” *J. Am. Chem. Soc.*, vol. 126, no. 9, pp. 2674–2675, 2004.

[4] D. D. Lasic and F. J. Martin, “On the mechanism of vesicle formation,” *J. Memb. Sci.*, vol. 50, no. 2, pp. 215–222, 1990.

[5] A. Jahn, W. N. Vreeland, D. L. Devoe, L. E. Locascio, and M. Gaitan, “Microfluidic directed formation of liposomes of controlled size,” *Langmuir*, vol. 23, no. 11, pp. 6289–6293, 2007.

[6] A. Jahn, S. M. Stavis, J. S. Hong, W. N. Vreeland, D. L. Devoe, and M. Gaitan, “Microfluidic mixing and the formation of nanoscale lipid vesicles,” *ACS Nano*, vol. 4, no. 4, pp. 2077–2087, 2010.

[7] R. R. Hood, C. Shao, D. M. Omiatek, W. N. Vreeland, and D. L. Devoe, “Microfluidic synthesis of PEG- and folate-conjugated liposomes for one-step formation of targeted stealth nanocarriers,” *Pharm. Res.*, vol. 30, no. 6, pp. 1597–1607, 2013.

[8] T. A. Balbino *et al.*, “Continuous flow production of cationic liposomes at high lipid concentration in microfluidic devices for gene delivery applications,” *Chem. Eng. J.*, vol. 226, no. September 2015, pp. 423–433, 2013.

[9] M. Mijajlovic, D. Wright, V. Zivkovic, J. X. Bi, and M. J. Biggs, “Microfluidic hydrodynamic focusing based synthesis of POPC liposomes for model biological systems,” *Colloids Surfaces B Biointerfaces*, vol. 104, pp. 276–281, 2013.

[10] D. Carugo, E. Bottaro, J. Owen, E. Stride, and C. Nastruzzi, “Liposome production by microfluidics: potential and limiting factors.,” *Sci. Rep.*, vol. 6, p. 25876, 2016.