LIPOSOME NANOPARTICLES SYNTHESIS USING 3D MICROFLUIDIC HYDRODYNAMIC FOCUSING

INTRODUCTION

Despite considerable progress in recent years, various diseases' diagnosis and treatment continue to present constraints, such as low sensitivity or specificity, drug toxicity, and severe side effects1. Cancer represents one of the best examples of a disease where localized delivery of therapeutics is of high importance, as the potent yet toxic mechanisms of action of such compounds can lead to an effective response or side effects. Today, most drug formulations are not capable of targeting specific sites of interest. Nanoparticle-based drug delivery platforms have emerged as suitable vehicles for overcoming these limitations [2]. Nanoparticles, such as liposomes, have proven advantageous at preserving therapeutic material and allowing for extended half-lives of drugs within the body[3].

Liposomes were discovered in the 1960s. These hollow nanoparticles are phospholipid vesicles consisting of at least one lipid bilayer (figure 1). This bilayer is usually composed of amphiphilic phospholipids that have a hydrophilic phosphate head and a hydrophobic tail consisting of two fatty acid chains. This structural feature has facilitated liposomes' applications, including their use as artificial cell membranes, carriers for drug delivery systems, encapsulating agents for food ingredients, and analytical tools [4–8].

During the COVID-19 pandemic, the first vaccines to reach clinical trials were based on viral vector and nucleic acid technologies. One of the most promising vaccine candidates was based on nucleoside-modified mRNA and encapsulated within liposome nanoparticles [9]. This only confirms the need for liposome nanoparticles for present and future drug delivery applications.

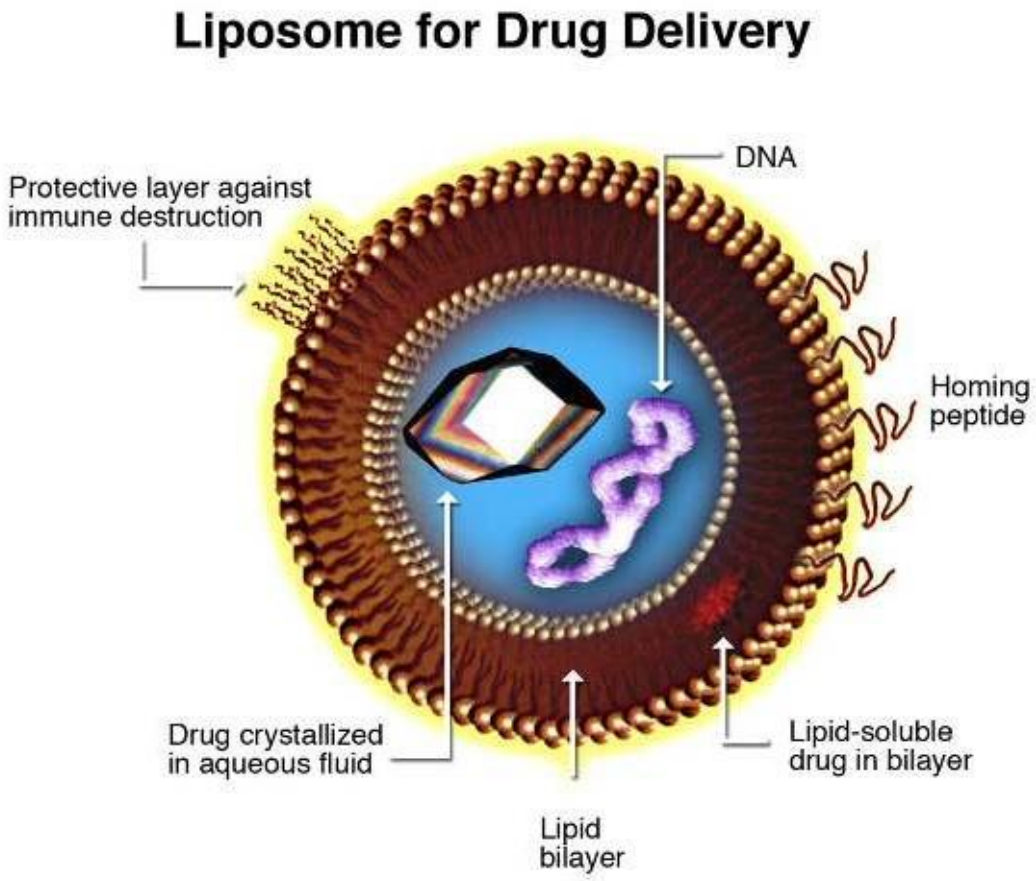


Figure 1: An example of a lipid nanoparticle (LNP) composed of phospholipids, homing peptide, drugs, and nucleic acids.

The size of these nearly spherical lipid vesicles can range from a few nanometers to several micrometers. However, liposomes applied to medical use typically range between 50 and 450 nm[1].

i. Microfluidic approach for highly-controlled liposome production

Size and size distribution are essential properties that determine the clinical successes of the nanocarriers. Researchers have observed that size has an impact on liposome accumulation and penetration efficiency at a targeted tumoral site. [1] In the past, different methods have been proposed for the production of liposomes. Macroscale batch methods are widely employed for liposome production (e.g. sonication technique, hydration method10), or extrusion method [11]. These techniques often show broad size distribution, and inconsistent encapsulation efficiency10, and are inadequate if one requires controllability and reproducibility.

Recently, improvements have been made with the development of microfluidic production methods, in which liposome formation occurs within a confined microenvironment. Using micrometer channels of varying diameters (typically up to 500 µm), fluid streams result in laminar flow. In a typical microfluidic liposome production system, an organic phase (ethanol, isopropyl alcohol, etc.) containing phospholipids flows in parallel with an aqueous phase (distilled water, PBS, etc.). This results in diffusive mixing and local dilution of the organic phase. The organic phase concentration decreases until it reaches solubility limit of the lipids. This triggers self-assembly of phospholipids into liposomes, that are subsequently collected. Under microfluidic conditions, it is possible to precisely tune parameters, such as aqueous and organic flow rates, to achieve different liposome sizes and encapsulation efficiencies. These methods have demonstrated higher control over the physical properties of the end product, particularly in terms of liposome size and size distribution. [12]

	Standard batch method	Fluigent microfluidic method
Particle size distribution	Low	High
Reproducibility	Low	High
Live particle size control	No	Precise
Range of particle size	Limited size range	Wide size range
Continuous (/in line) production	No	Yes

Table 1: Comparison of liposome synthesis specification between standard batch method, and Fluigent microfluidic method

ii. A glass capillary device for improved particle size distribution

Many microfluidic systems make use of the microfluidic hydrodynamic focusing (MHF) technique to synthetize liposomes. In standard designs (two-dimensional (2D) MHF), an organic phase with lipids flows in the center of a rectangular channel, and an aqueous buffer flows on both sides of it (figure 2A). However, as 2D-MHF chips rely on a planar flow-focusing configuration, they show a poor surface to volume ratio, limiting liposome synthesis at the two lipid/aqueous interfaces. Side effects are also encountered at the upper and lower parts of the chip. These designs usually require specific surface coatings depending on the fluids used. To overcome these limitations, glass capillary devices have been developed to generate a hydrodynamically focused 3D stream, where the aqueous phase fully surrounds the lipid phase (figure 2B). As a result, radially symmetric mixing of the fluidic inputs is realized, facilitating the production of highly uniform nanoscale liposomes. This design has shown narrower size distribution compared to 2D-MHF13. These devices usually do not require any surface coating as there is no contact between the lipidic phase and the wall of the capillary in which the liposomes are formed.

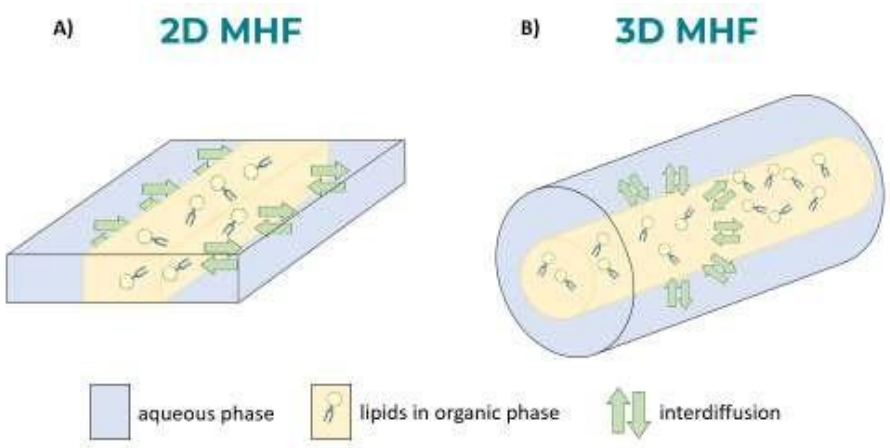


Figure 2: Two microfluidic designs for liposome synthesis. A) two-dimensional microfluidic hydrodynamic focusing (2D MHF) showing mixing at the two lipid/water interfaces and B) three-dimensional microfluidic hydrodynamic

In this application note, we show highly monodisperse liposome synthesis using the Raydrop™(Raydrop™ is a registered trademark of Secoya Technologies): a commercially-available glass capillary-based microfluidic device, with 3D axisymmetric geometry.

MATERIALS AND METHODS

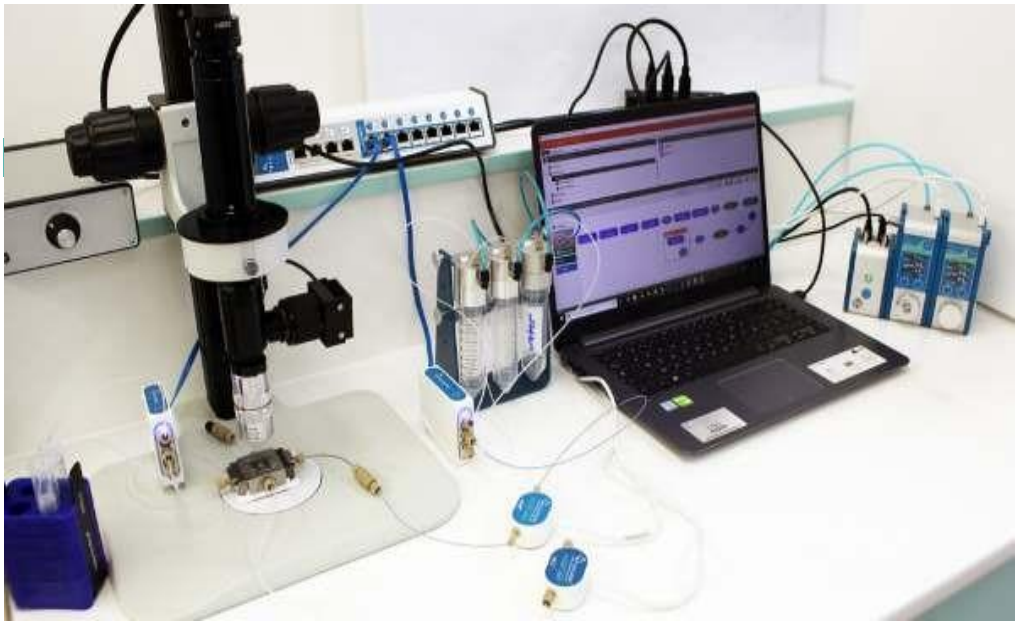


Figure 3: Setup for liposome synthesis

I. Materials

i. Microfluidic system

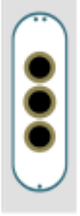
Pressure controller

Two **Fluigent Flow EZ** with 2 bar are used in the setup presented here.

Flow sensor

The **Flow Unit** is a flow sensor that allows real time flow rate measurement. By combining a Flow Unit with the Flow EZ, it is possible to switch from pressure control to flow rate control, allowing for the generation of highly monodispersed droplets over a long period of time. Two Flow Unit M are used to monitor and control the flow rates of the dispersed and continuous phase during the run.

2-Switch



A **3-port/2-way microfluidic valve** is used to switch between PBS and lipid + EtOH solution. This allows for better reproducibility by priming the system and adds a cleaning step during the experiment.

The RayDrop is developed and manufactured by Secoya

Microfluidic device

The **Raydrop™** is a versatile microfluidic device that can be used for highly-precise fluid mixing. It is based on the alignment of two glass capillaries immersed in a pressurized chamber containing the outer phase. The inner phase exits the capillary through a 3D-printed nozzle, placed in front of a second capillary, where it is surrounded by the outer phase. This non-embedded implementation of axisymmetric flow-focusing is referred to co-flow-focusing, and allows one to generate a hydrodynamically focused 3D stream. The advantage lies in his geometry that prevents wettability issues that could appear in other microfluidic chips. This allows for the generation of highly monodispersed nanoparticles with any type of fluid.

ii. Reagents

Inner phase

Absolute ethanol (LiChrosolv Supelco, Merck, Germany), Lipids 10 mg/mL (Phospholipon 90 G, Lipoid, Switzerland), and Dimethyldioctadecylammonium (DDAB) 2.5 mg/mL (from Merck, Germany) filtered with 0,2 µm syringe filter.

Outer phase

Phospate-buffered saline (PBS) (pH 7.2) filtered with 0,2 µm syringe filter.

Inner phase for priming and cleaning

Absolute ethanol (LiChrosolv Supelco, Merck, Germany) filtered with 0,2 µm syringe filter.

II. Liposome synthesis and size analysis

As mentioned previously, a stream of lipid in alcohol solution is surrounded by an aqueous phase within a glass capillary. The alcohol solution containing lipids diffuses into the aqueous solution (and reciprocally the water diffuses into the alcohol), until the alcohol concentration decreases to the solubility limit of the lipids. As a consequence, this diffusion triggers the formation of liposomes by a mechanism described as “self-assembly”, where lipids assemble into a more energetically favorable structure. In microfluidic liposome synthesis, two parameters are usually utilized for tuning the size of the focused stream: the flow rate ratio (FRR) between the aqueous and the alcohol phases, and the total flow rate (TFR). Jahn et al. first demonstrated that the mean size of the liposome produced is inversely proportional to FRR. Conversely, a small effect on liposome size is observed when changing the TFR. [14,15] These results were confirmed in additional studies. These parameters are utilized in this application note.

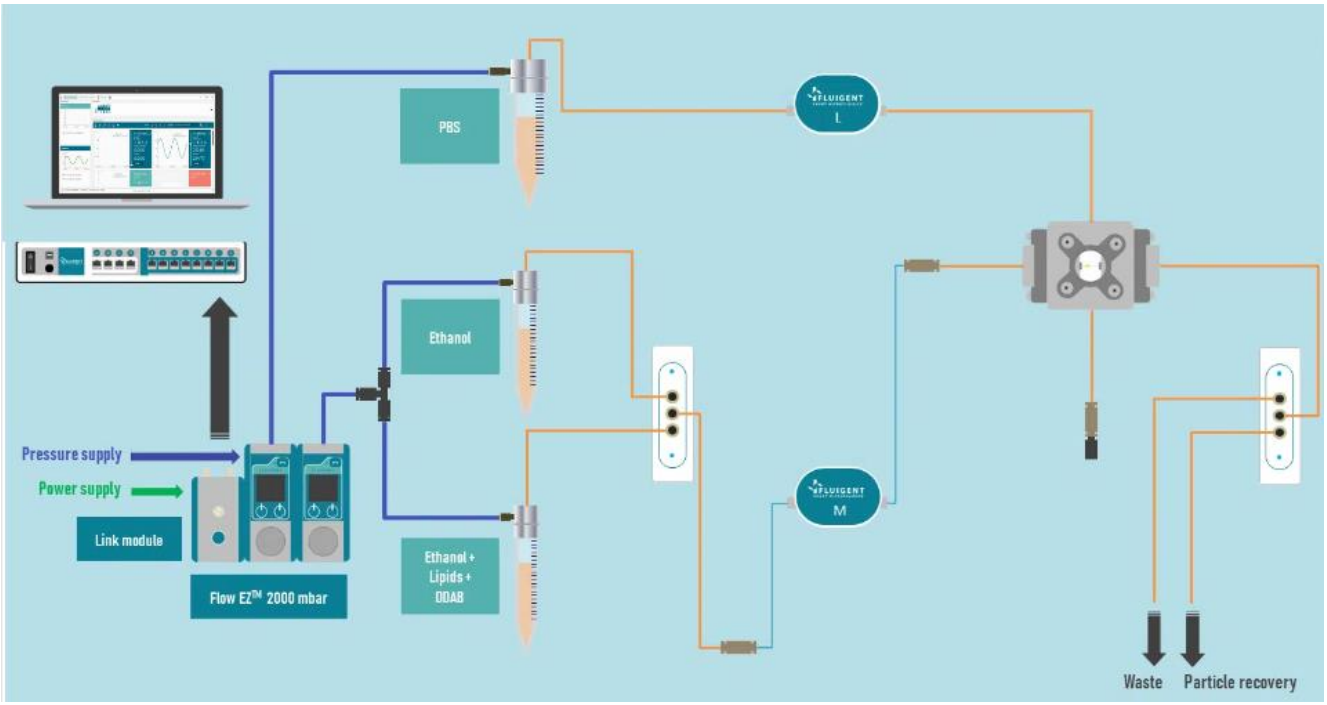


Figure 4: Scheme for liposome synthesis

The liposome production system is illustrated in figure 4. Two reservoirs of 15 mL containing absolute ethanol, and lipids in ethanol are connected to a 2-SWITCH, which is connected to the microfluidic device via 1/16 in. PEEK tubing (inner phase). One reservoir of 50 mL containing PBS is connected to the second inlet of the device (outer phase). The tubing passes through flow units allowing flow rate measurement and control. The outlet tubing can be connected to a second 2-SWITCH to switch between waste and particle recovery.

i. Liposome synthesis

1. The 15 mL reservoir containing absolute ethanol is selected by the 2-SWITCH. Ethanol is injected as inner phase. At the same time, PBS (surrounding outer phase) flow is started to prime the system and generate a co-flow of ethanol and PBS. Identical pressure should be applied on both reservoirs.
2. As soon as a steady co-flow is reached, the solution of lipids in ethanol can be easily injected by switching the 2-SWITCH to the second position (lipids/ethanol).
3. Liposomes should be produced in less than 1 min (time for the liquid to switch)
4. Pressure and/or flow rate can be then adjusted to target the stream diameter (figure 5), desired TFR and FRR, and the related liposome size.

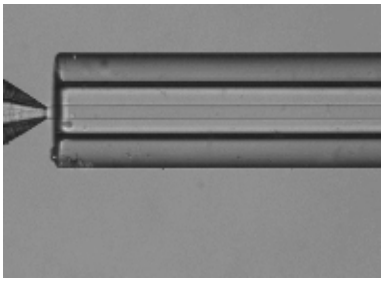


Figure 5: A liquid stream of ethanol with lipid, surrounded by PBS

5. To stop the experiment, identical pressure is applied on both reservoirs. Absolute ethanol is injected by switching the 2-SWITCH to first position (at 10 µL/min the liquid replacement takes approx. 70 s, depending on the tubing used). The dispersed phase is then stopped, and the tubing at the injection entry is removed to allow for the injection capillary to be filled with the aqueous phase. Pure water should be used instead of PBS for cleaning.

For more information about assembly, filling and cleaning using the Raydrop™, please refer to the Good practice guide on droplet generation process.
In this application note, we determine the effects of the FRR and the TFR on liposome size. Table 2 summarizes the flow rates used.

Q_{PBS} $[\mu L/min]$	$Q_{lipids/EtoH}$ $[\mu L/min]$	TFR $[\mu L/min]$ $= Q_{lipids/EtoH} + Q_{PBS}$	FRR $[-]$ $= Q_{PBS}/Q_{lipids/EtoH}$
90	10	100,00	9,00
80	20	100,00	4,00
95	15	110,00	6,33
90	25	115,00	3,60
190	30	220,00	6,33

Table 2: Summary of the stream generated using different flow rates

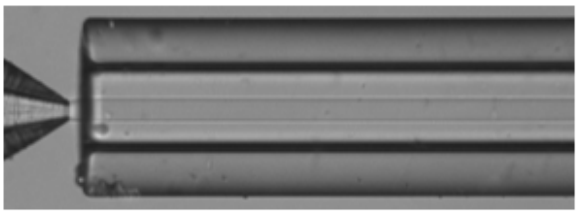
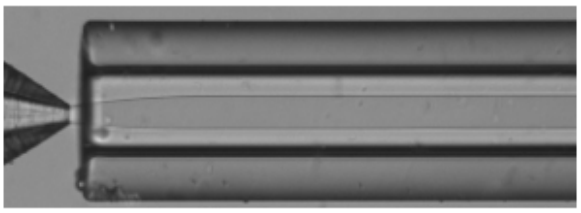
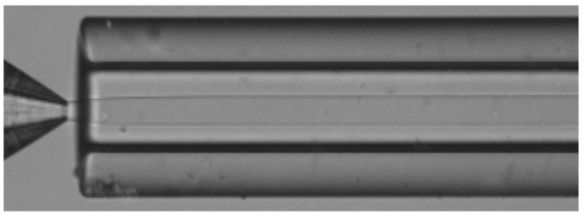
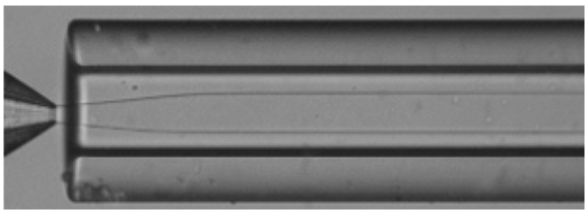
ii. Liposome size analysis

Analysis using a Dynamic light scattering (DLS) system (Nanoflex 180, Microtrac, USA), assuming n=1.45 for liposomes, n =1.33 for the water phase (PBS) and a spherical shape for the liposomes. For each sample, 3 measurements of 60 s each are performed and the mean value is calculated.

RESULTS & DISCUSSION

Table 2 shows the used pressures, flow rates, TFR, FFR and the corresponding generated inner stream diameters of the lipids/ethanol solution. We observe from the images a well-centered and steady inner flow, confirming enhanced flow stability using the Raydrop™. We also note from the same table that the stream diameter increases when the FRR increases. Lipid solubility limit is reached, and liposomes are synthesized.

Figure 5: A liquid stream of ethanol with lipid, surrounded by PBS

Dispersed phase (lipid/EtOH)		Continuous phase (PBS)		TFR [µL/min]	FRR [-]	Stream diameter [µm]	Micrographs
P (mbar)	Q (µL/min)	P (mbar)	Q (µL/min)				
300	10	200	90	100	9,00	39	
600	20	200	80	100	4,00	59	
445	15	200	95	110	6,33	49	
792	25	200	90	115	3,60	65	
1019	30	380	190	220	6,33	52	

Once synthetized, liposome size analysis is performed using DLS. For each sample, 3 measurements were performed, and the median diameter is calculated, associated with its polydispersity index (measure of the distribution). Figure 6 shows the liposome median diameter and its corresponding polydispersity index as a function of the FRR. TFR was kept constant around 100 µL/min. We observe that liposomes with a diameter ranging from ~30 nm to ~155 nm were generated under these conditions. In addition, we observe that when the FRR increases, the liposome diameter decreases. In fact, a diameter of 87 nm is obtained using a FRR of 4, while a diameter of 29 nm is obtained using a FRR of 9. This result is in good agreement with the literature using microfluidic methods [14,15], and is likewise attributed to the increasing degree

of focusing experienced by the center stream of solubilized lipid [13]. This level of control on the liposome diameter is not possible to reach using standard batch methods. Depending on the parameters used, a wide range of liposome sizes is demonstrated. This size range achieved is wider than typical alternative microfluidic systems [13]. From the same figure, we observe that the PDI ranges from 0,07 to 0,15.

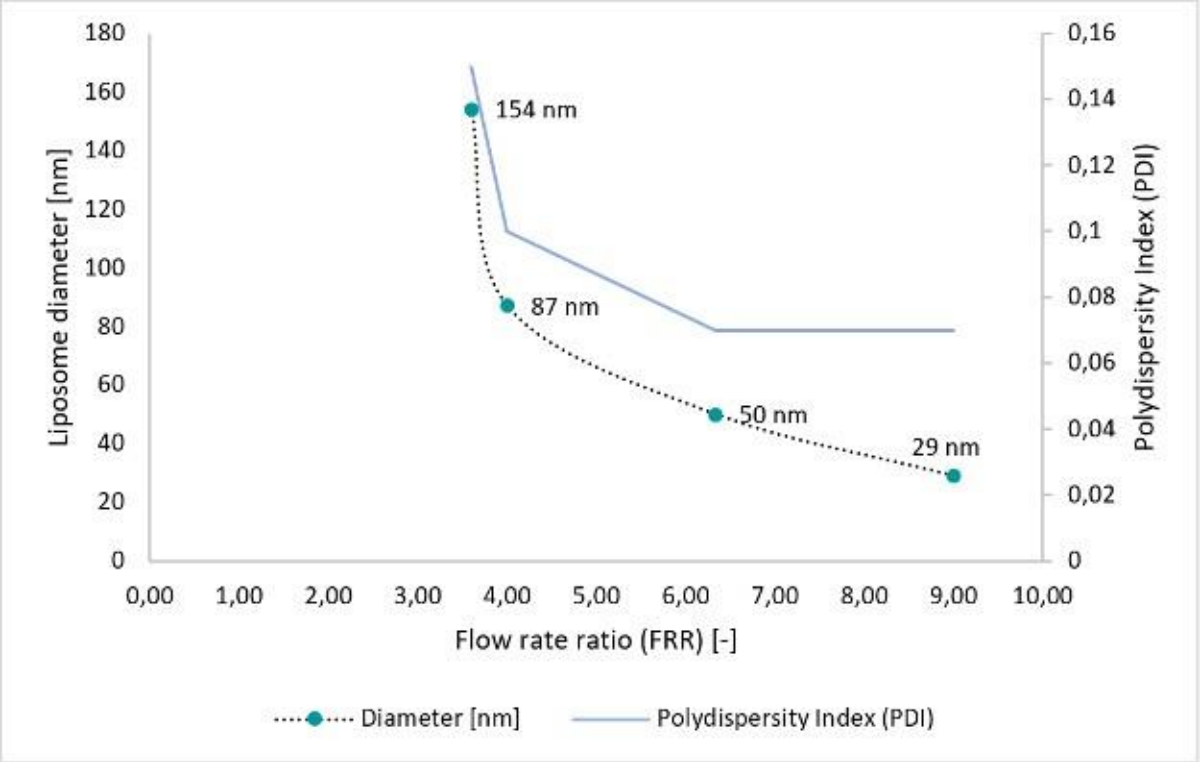


Figure 6: Liposome mean diameter and polydispersity index (PDI) as a function of the flow rate ration (FRR)

Finally, the impact of the TFR on liposome size was investigated. The FRR was kept constant using around 6,33, while varying the TFR. Table 4 shows the liposome diameter and PDI as a function of the TFR.

$\boldsymbol{TFR}$ $[\mu L/min]$	$\boldsymbol{FRR}$ $[-]$	$\boldsymbol{Liposome\ diameter}$ $[\mu m]$	$\boldsymbol{PDI}$
110,00	6,33	50	0,07
220,00	6,33	59	0,15

Table 4: Liposome diameter and related PDI as a function of the TFR. The FRR is kept constant around 6,33

We observe that by doubling the TFR from 110 µL/min to 220 µL/min the liposome diameter varies from 50 to 59 nm. This result is also in good agreement with the previous studies. [14,15]

The generation of highly monodisperse liposomes at sizes ranging from approximately 30 nm to 150 nm were generated, confirming the great potential of the Raydrop™ for liposome synthesis. It is worth noting that it is possible to reach a broader liposome size range by using other values of FRR.

CONCLUSION

Liposome nanoparticles prove advantageous at solubilizing therapeutic substances. Macroscale batch methods widely employed for liposome production lack control on liposome morphology, size, and distribution. Microfluidic systems allow for the production of highly monodisperse liposome nanoparticles. We have demonstrated the production of liposomes using a microfluidic system consisting of pressure-based flow controllers and the Raydrop™ microfluidic device with standard configuration. Liposomes ranging from 30 to 150 nm were generated. Sizes can be adjusted by controlling the device flow input parameters, particularly the flow rate ratio (FRR). The polydispersity index (PDI) ranges from 0,07 to 0,15. This system enables the synthesis of liposomes for drug delivery applications, as encapsulating agents for food ingredients, or for other applications requiring nano-sized and spherical liposomes.

A complete, cost-effective, and commercially-available platform for the on-demand production of monodisperse liposome nanoparticles is now available. This allows for control of liposome size and frequency by adjusting flow parameters.

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