DESIGN AND FABRICATION OF FLUIDIC DEVICES FOR INDUSTRIAL-SCALE PRODUCTION OF LIPID-BASED NANOPARTICLES AND BIOMOLECULES ENCAPSULATION

by

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Conception et fabrication de dispositifs fluidiques pour la production à l'échelle industrielle de nanoparticules à base de lipides et l'encapsulation de biomolécules

Fatemeh BEHROUZ

RÉSUMÉ

Les nanoparticules de liposomes sont produites à l'aide de techniques descendantes et ascendantes, et elles sont utilisées comme vecteurs de délivrance dans l'administration de médicaments, des protéines, des étiquettes et des applications de matériel génétique. L'augmentation du débit et de la reproductibilité des liposomes est essentielle pour la production à l'échelle industrielle de liposomes afin de les considérer comme des nanomédicaments qui pourraient être possibles en utilisant des micro-mélangeurs ainsi que des milli mélangeurs. Les mélangeurs à l'échelle micrométrique (micromélangeurs) génèrent une nano précipitation qui est l'une des approches ascendantes les plus réussies et peut contrôler les propriétés des liposomes telles que leur taille et leur distribution de taille. D'autre part, les micro-canaux à l'échelle millimétrique (milli mélangeurs) sont capables de produire des liposomes à l'échelle nanométrique, par lesquels la production de liposomes à des taux élevés est autorisée en raison de la plus grande dimension de la section transversale. En outre, l'élimination des résidus toxiques est un autre défi lié à l'approche mentionnée. Dans ce travail, différentes conceptions de production de dispositifs fluidiques sont explorées à l'aide de la simulation numérique et des techniques de fabrication modernes. Le contrôle de la taille, de la distribution de taille et du potentiel zêta (ZP) des liposomes, ainsi que de leur toxicité, de leur pureté et de leur taux de production, se fait en caractérisant et en évaluant les liposomes synthétisés. Le transport de médicaments vers des emplacements cibles à l'intérieur du corps humain serait possible en contrôlant la taille des liposomes produits. La production de différents vaccins en particulier pour les vaccins Covid-19 par encapsulation d'ARNm est l'une des applications les plus populaires pour les liposomes à taille contrôlée.

Mots clés: liposomes; micromélangeur; millimélangeur; haut débit de production; nanoparticules à faible toxicité.

This summary was enhanced for translation to French using AI (ChatGPT).

Design and fabrication of fluidic devices for industrial-scale production of lipid-based nanoparticles and biomolecules encapsulation

Fatemeh BEHROUZ

ABSTRACT

Liposome nanoparticles are produced using top-down and bottom-up technics, and they are used as delivery carriers in drug delivery, proteins, labels, and genetic material applications. Increasing liposome throughput and reproducibility are essential for industrial-scale production of liposomes in order to consider them as nanomedicines that could be possible using micromixers as well as milli mixers. Micrometer-scale mixers (micromixers) generate nanoprecipitation which is one of the most successful bottom-up approaches and can control properties of liposomes such as their size and size distribution. On the other hand, millimeter-scale microchannels (milli mixers) are able to produce liposomes of nanometric scale, by which the production of liposomes at high rates is allowed because of the larger cross-section dimension. Besides, the reducing of toxic residues is another challenge related to the mentioned approach. In this work, different designs for producing fluidic devices are explored using numerical simulation and modern fabrication techniques. Controlling liposomes size, size distribution, and zeta potential (ZP), as well as their toxicity, purity and production rate, is done by characterizing and evaluating synthesized liposomes.

Keywords: liposomes; micromixer; milli mixer; high throughput; low toxicity nanoparticles.

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LIST OF ABREVIATIONS

CHOL	Cholesterol
DHP	Dicetyl phosphate
DLS	Dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DS	Delivery system
FRR	Flow rate ratio
GUV	Giant unilamellar vesicles
LET	Liposomal encapsulation technology
LNPs	Liposome nanoparticles
LUV	Large unilamellar vesicles
ME	Mixing efficiency
MHF	Microfluidic hydrodynamic focusing
MHF-LC	Microfluidic hydrodynamic focusing large channel
MLV	Multilamellar vesicles
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffer saline solution
PDI	Polydispersity index
PDM	Periodic disturbance mixer
PDMS	Polydimethylsiloxane
SHM	Staggered herringbone micromixer
SUV	Small unilamellar vesicles
TEM	Transmission electron microscopy

TFF	Tangential flow filtration
TFR	Total flow rate
VFF	Vertical flow focusing
ZP	Zeta potential

LIST OF SYMBOLS

μ	Dynamic viscosity
A	Lipid area
c	Concentration
D	Diffusion coefficient
De	Dean number
D_h	Hydrodynamic diameter
F	External force
k	Boltzmann constant
N	Molar flux
n	Number
NA	Avogadro's number
p	Pressure
P	Production rate
Q	Total flow rate
Q_{as}	Aqueous solvent flow rate
Q_{os}	Organic solvent flow rate
rh	Particles Ratio or Liposome Ratio
R	Volumetric source for the species
Re	Reynolds number
T	Temperature
u	Flow velocity

x	Distance
η	Viscosity
ν	Kinematic viscosity
ρ	Fluid density
σ	Standard deviation

INTRODUCTION

One of the greatest challenges during the COVID-19 pandemic was the vaccines production rates. Unfortunately, this slow and time-consuming process led to many people losing their lives. But after distribution of vaccines the situation has taken under control which took about two years. Considering the COVID-19 pandemic, if a similar crisis occurs again, what would be the consequences of delaying in vaccine production? In this thesis, a technology has been introduced to effectively boost vaccine production and drug delivery capacity.

Liposomes have attracted attentions significantly in the race for producing COVID-19 vaccines. These sphere-shaped vesicles that consist of a lipid bilayer, are crucial in therapeutics delivery applications as they are generated by self-assembling and their structure contain a hollow, which protects important molecules, especially in mRNA vaccines for COVID-19, gene delivery applications and artificial cell membranes (R. R. Lopez et al., 2020; Maeki, Kimura, Sato, Harashima, & Tokeshi, 2018).

They also are able to do target delivery of the encapsulated components to specific sites and effectively protecting delicate molecules (López, 2020). Drug delivery application is a growing market and driven by the requirement for targeted delivery for chronic disease and the need for advancement in technology to sale up the liposome production. In addition to that, a significant concern in liposome production is reproducibility of the product while meeting the criteria of regulatory agencies (López, 2020).

Compared to conventional methods, continuous flow conditions in fluidic devices increase liposome reproducibility and simultaneously can scale liposome production with controlled liposome size and tunable physical properties of vesicles**.** Micromixers are one of the fluidic devices in which mixing channel diameter is in the micrometer scale. The yield of micromixers normally is not suitable for mass production on an industrial scale. Fabrication of the micromixers with higher yield is also challenging and requires high manufacturing costs due

to parallelization and the production of multiple chips. Milli mixers (or millimeter-scale flow mixers) are another type of fluidic device that has been proposed as a scalable and cost-effective route to produce larger amounts of liposomes with size and polydispersity index (PDI) more relevant to therapeutic applications.

The application of liposomes in drug delivery can revolutionize the pharmaceutical industry through improving drug management and drug delivery. The global market for this technology is growing substantially over the next decade due to the key role that liposomes have in medical treatments, especially in tumor-targeted therapy. The valuation of the global cancer therapeutics market for liposomes by 2022 was USD 164 billion. As we can see in Figure 0.1, the growth in the funding for cancer treatment is advancing for fluidic systems, thereby enhancing the efficiency of drug delivery and reducing the costs related to the production process are important.

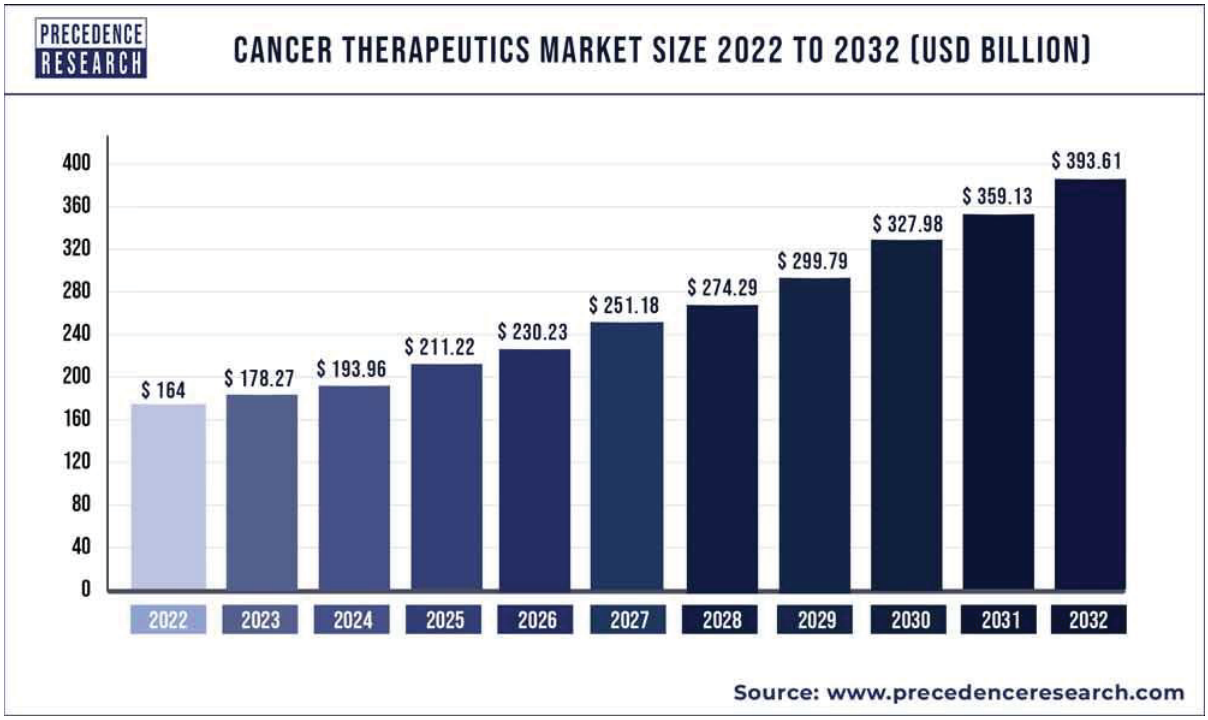


Figure 0.1 Global Cancer Therapeutics Market Size Growth taken from (precedenceresearch, October 2023)

As we can see in Figure 0.1, the market for cancer therapeutics is anticipated to grow around three times by 2032. To respond to the high demand of liposomes in the near future, it is important to develop efficient mixers for increasing the production scale while controlling the synthesis process. It is possible to produce liposomes with current batch-based methods but there are some problems with those methods such as lack of continuous production, reducing toxicity, and problems in controlling liposome size. These issues were addressed previously using methods such as parallelizing microfluidics (López, 2020). The problem with this method is that producing and parallelizing ten micromixers is expensive and time consuming. Therefore, it is important to introduce a method which can solve the problem of mass production while it remains cost effective.

The aim of doing this research project is to achieve mass production of liposomes by scaling up the micromixers and subsequently produce milli mixers to increase liposome production rate while adhering to industry standards to reduce waste and prevent parallelizing many micromixers for mass production of liposomes. The production conditions and factors controlling liposome physicochemical characteristics such as size distribution or poly dispersity index, size, and zeta potential or surface charge of the particles should be investigated using different technics such as Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS). The significance of this thesis and research objectives are provided in the following to further illustrate the research project.

Problematics

Mass production of liposomes is one of the most critical challenges for scientists, affecting liposome usability in the industry, vaccination, and healthcare system. Besides, the fabrication and parallelization of current micro fluidic devices is laborious and material and time consuming. Additionally, the functionality of the liposomes is highly dependent on their physicochemical characteristic, and those characteristics can be controlled by changing and managing the synthesis conditions. Manufacturing a cost-effective device that can increase

liposome throughput without negative influence on the final liposome physicochemical characteristics is required to answer this challenge.

Objectives

The objective of this project is to increase the production rate of liposomes using fluidic devices. The characteristics of the produced liposome nanoparticles and the factors influencing these properties would be evaluated to generate liposomes more adaptable with the industrial scale controls and healthcare system requirements for using in vaccine production and drug delivery application. The production of engineered liposomes at a high production rate with specific characteristics not only would bring benefits to the scientific community and pharmaceutic companies in Canada and all medical drug delivery applications such as in vaccination internationally which conclude in saving many peoples' lives.

Three scaled-up milli mixers and different synthesis conditions such as three different TFRs, three different FRRs and three different amounts of Phosphate-Buffered Saline (PBS) are used to control the physicochemical characteristics of liposomes to produce suitable liposomes for drug delivery applications. We hypothesize that increasing the dimensions of the mixing channel can affect liposomes' size and polydispersity index because of changes may happen in the mixing patterns inside the channel. But the zeta potential is considered to be influenced by varying the amount of PBS as it is ionic solvent and can influence the surface charge of the particles. This work's main contribution is mass production of liposomes while controlling their physiochemical parameters and reducing toxicity.

Chapter 1 investigates the literature on the primary components of this project: liposomes and micromixers and increasing liposome throughput. Chapter 2 reviews materials and methods used for fabricating and designing milli mixers, liposome synthesis and characterization and all the required steps in this process. Chapter 3 delves into describing the design and simulation results of the scaled-up milli mixers and obtaining mixing efficiency of the mixing channel.

The results of mixing efficiency of scaled-up PDM milli mixer is compared with PDM micromixer by simulation and visualization as well. In Chapter 4, the focus was on investigation of the parameters influencing liposome phytochemical characteristics to control over the size, PDI and zeta potential of the liposomes. And finally in Chapter 5, a comparison is made between the liposomes synthesized using PDM milli mixer in this project and the liposomes synthesized using PDM micromixer by Lopez et al. (2021).

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Liposomes have been used in several different applications such as in drug delivery systems especially for tumor treatment (Allen & Cullis, 2013; R. R. Lopez et al., 2020), gene delivery (dos Santos Rodrigues, Banerjee, Kanekiyo, & Singh, 2019), encapsulating agents in the food industry (Shukla, Haldorai, Hwang, Bajpai, Huh, & Han, 2017), and artificial cell models (Cans et al., 2003), since they constitute the biggest clinically established nano-scale systems in a decade-long clinical presence (Al-Jamal & Kostarelos, 2007). In this chapter, we will go through a literature review about liposomes and highlight the applications and importance of these nanoparticles. Then different liposomes production methodologies are discussed and investigated to find the best way of producing these carriers to further promote their applications in the field of healthcare.

1.2 Liposomes

Liposome nanoparticles are made of lipids with an amphiphilic nature. Lipids have one hydrophilic head and two hydrophobic tails. A wide variety of lipid types are available that can be used together as different liposome formulations. Selecting lipids is the first step in liposome synthesis. Some of the lipid types for liposome production include dicetyl phosphate (DHP), phosphatidylethanolamine (PE), polyethylene glycol (PEG) dihexadecyl phosphate (DCP), phosphatidylcholine (PC),1,2-dimyristoylphosphatidylcholine (DMPC), 1,2-diplmitoylphosphatidylcholine (DPPC), 1,2-disteroylphosphatidylcholine (DSPC) and cholesterol (CHOL) (Hood & DeVoe, 2015; Huwyler, 2018; R. R. Lopez et al., 2020). In the second step, lipids should be formulated and diluted in an appropriate organic solvent. Organic solvents with different characteristics are available including ethanol, IPA, methanol, and Transcutol®, which should be selected based on the lipid solubility (Joshi et al., 2016). Then

the solvent should be removed totally by dialysis but in this work, we didn't use dialysis for the liposomes because we wanted to compare the results of synthesis of liposomes in milli mixers to the results of liposomes synthesized in micromixer.

In the third step, the solution is mixed with an aqueous phase (usually PBS, which is Phosphate-Buffered Saline, 10 mM, pH 7.4.). Then, lipids come together to form disk-shaped lipid bilayer structures. From an energy point of view, on the edges of these structures, the lipids exposed their hydrophobic tails to water, creating edge energy (Lasic, 1988; Lasič, 1987). By growing these discs, to reduce edge energy, they will bend and close to form spherical vesicles with hydrophilic nature inside and outside them, and lipophilic nature within their bilayers, and a size range from 20 nm to 2 μm. Once the vesicles are formed, the overall energy decreases which traps the liposomes in a high energy state. In micromixers, the energy needed for liposome formation is provided by changes in the medium polarity. The changes in the polarity happen because of the rapid mixing that happens by pumping the fluid into the mixing channel (López, 2020). In fact, in the passive micromixers, the mixing is happening by just relying on the fluid dynamics inside the microchannels without any external energy source, to make the required changes in polarity.

Liposome physicochemical properties such as size (hydrodynamic diameter), polydispersity index (PDI), and zeta potential are determinants in liposome applications and their interaction with the human body. (R. R. Lopez et al., 2020)

Liposome size is one of the important factors in determining liposome applications. Tuning liposome size between 50-200nm is beneficial, since this range is appropriate for liposome formulations in the market (Huwyler, 2018). PDI or size distribution values especially in drug delivery applications should be low. Monodisperse populations have size distributions with PDI < 0.1, while PDI < 0.2 indicates low polydispersity suitable for pharmaceutical applications (Hood & DeVoe, 2015).

Zeta potential is the net electric charge of the particle that determines the interaction between liposomes and cells. Zeta potential values vary from -55 mV to 62 mV (R. R. Lopez et al., 2020). It is indicated in in-vivo models that the positive values of zeta potential can increase circulation time and help with the applications of liposomes in delivery systems (DS). Using the ZetaPlus equipment the measurements of zeta potential values could be obtained at 25 °C and a pH = 7.00 (R. R. Lopez et al., 2020).

According to the investigations, there is a relationship between flow conditions and liposome size, size distribution, and zeta potential. Flow conditions in fluidic devices are defined by flow rate ratio (FRR), which is a ratio between the aqueous phase and organic phase, and total flow rate (TFR), which is the total speed of aqueous and organic streams passing through the microchannels. The relationship of FRR and TFR with size, PDI, and zeta potential were modeled, which indicated that by modifying TFR and FRR, liposome size and PDI can be tuned in a way that by increasing FRR, liposome size and PDI values decreased (R. R. Lopez et al., 2020; Tabrizian, 2019). TFR and FRR didn't affect zeta potential values, but only composition could change its values (Huwyler, 2018; Zouggari, 2022).

Liposomes have a hollow inside their structure that helps them with encapsulation of drugs and other components to carry inside the human body (Akbarzadeh et al., 2013). They proved to be suitable for delivery system (DS) due to their ability of drug encapsulation. The entrapped compounds could be released at designated targets without any decomposition (Akbarzadeh et al., 2013). They form a resistant barrier to enzymes around the contents inside them, so the contents would be prevented from oxidation and degradation. Therefore, they are known as a good candidate for gene delivery application, since they can protect genetic material against enzymes (Fraley, Subramani, Berg, & Papahadjopoulos, 1980). Furthermore, co-loading of drugs is possible that opens the door to custom therapies. While this does not affect entrapment efficacy, it can enhance the speed of drug release compared to liposomes containing only one drug. (Joshi et al., 2016).

Liposome-cell interactions and their fate inside the human body after administration determine their limitations and benefits. According to the in vivo and in vitro studies, the interactions of

liposomes with cells are classified in four categories which are presented in Table 1.1 (Akbarzadeh et al., 2013).

Table 1.1 Cell-liposome interactions

Cell-liposome interactions	**Explanation of interaction**	**portion**
Simple absorption	Interactions with cell-surface components, electrostatic forces, or by nonspecific weak hydrophobic	main
Endocytosis	Phagocytic cells i.e., macrophages and neutrophils	main
Exchange of bilayer components	Transferring of lipids between the two vesicle types	possible
Fusion with the plasma cell membrane	Lipid bilayer of the liposome would be inserted into the plasma membrane and the liposomal content releases into the cytoplasm simultaneously	rare

1.2.1 liposome classification

Liposomes' circulation half-life is determined by the vesicle size. The size and number of bilayers are determinants in drug encapsulation capacity. Liposomes can be classified into two categories according to their size and the number of bilayers. It is displayed in Figure 1.1 that based on the number of bilayers, the liposome may have one bilayer that is named as a unilamellar vesicle, which has only one phospholipid bilayer. It may have more bilayers, which are called multilamellar vesicle (MLV) that has an onion shape structure or multivesicular vesicle, which is encapsulated by smaller vesicles. Based on the size, liposomes can be classified into three groups: (1) large unilamellar vesicles (LUVs), (2) small unilamellar vesicles (SUVs), and (3) giant unilamellar vesicles (GUV) (Akbarzadeh et al., 2013; van Swaay, 2013)

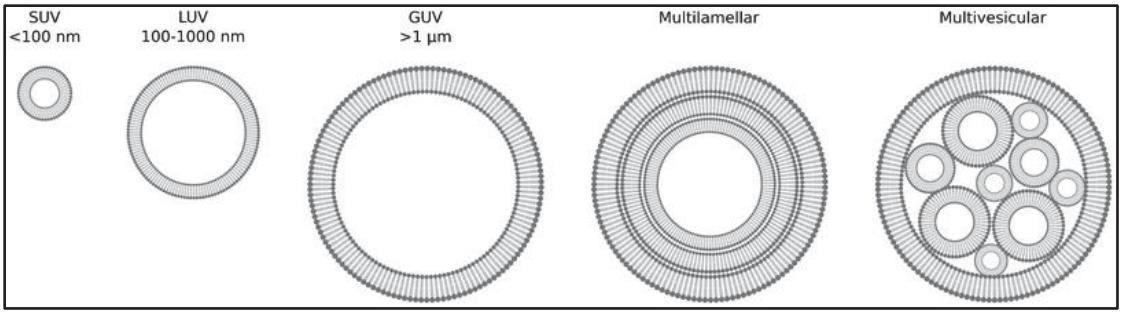


Figure 1.1 Classification of liposomes according to their size and the number of bilayers
Taken from van Swaay & deMello (2013)

1.2.2 Liposome production methods

Liposome production methods are various for laboratory scale production. There are different conventional methods and most of them use batch technics. Conventional liposome production methods involving thin-film hydration, crossflow injection, ethanol injection, and reverse evaporation usually produce polydisperse liposomes, and need homogenization steps such as sonication and extrusion (R. R. Lopez et al., 2020). Also, these methods have low reproducibility; thus, they require further post-production steps and complex scale-up to produce liposome nanoparticles with desired features (R. R. Lopez et al., 2020; Yanar, Mosayyebi, Nastruzzi, Carugo, & Zhang, 2020). Besides, the risk of toxicity of the final product is higher in these methods`, due to the remaining organic solvent residues in the liposome or aqueous phase(Mozafari, 2005).

On the other hand, microfluidic-based mixers produce liposomes with controllable size and ability for tuning properties of the demanded vesicles by controlling continuous flow (Yanar et al., 2020). Micromixers generate laminar flow to mix substrates in stable and uniform mixing conditions in millisecond time scale (Chen, Han, Shumate, Fedak, & DeVoe, 2019; Yanar et al., 2020). Micromixers can control liposome properties, and produce liposomes with diameters below 50nm suitable for drug delivery applications (Chen et al., 2019). Some of the disadvantages of micromixers including high manufacturing cost, complex device operation, low production rates, and the low durability of the device should be solved (Chen et al., 2019; R. R. Lopez et al., 2020; Yanar et al., 2020).

Millimeter-scale flow reactors (milli mixers) were produced as a solution to the problems of micromixers. These mixers consist of channels in a millimeter-scale that can increase particle production capacity, decrease the production cost, and control flow to tune particles' properties. Besides, they are good candidates for producing liposomes with compatible characteristics appropriate for medicinal applications. This demonstrates their ability for liposome mass production in a cost-effective way for pharmaceutical applications.

Considering these techniques for liposome production, liposomes produced by batch production methods have larger diameters and wider size distributions than the ones produced by the milli mixers with smaller size and narrower size distributions (Yanar et al., 2020).

1.3 Liposome production using fluidic devices

Fluidic devices offer an approach that helps to control liposome production variables more efficiently and optimize liposome properties such as size and size distribution. Fluidic devices in liposome production are classified into two main groups, micromixers and milli mixers.

1.3.1 Micromixers in liposome production

Micromixers are used as an alternative to conventional liposome production methods, which can promote the control of liposome properties to yield on-demand liposomes. Micromixers are generally based on molecular diffusion, chaotic advection, and Dean forces. The width of microchannels inside micromixers vary from 10 to 500μm. Laminar flow is prevalent at this scale, which makes stable and uniform mixing inside the microchannels in milliseconds (Cai, Xue, Zhang, & Lin, 2017)**.** Besides, laminar flow regimes make a slow diffusion process and limit mass transfer across the channel that is called 'microfluidic mixing challenge'. Different mixing strategies have been proposed to solve this challenge such as reducing channel length to achieve shorter mixing time in passive micromixers (Chen et al., 2019).

1.3.1.1 Active and passive micromixers

Micromixers are mainly classified as active and passive micromixers in terms of structure design. In active micromixers, there is an external energy source for operation besides fluid pumping energy, e.g., acoustic waves in acoustic micromixers. In this term, a high number of nanoparticles were synthesized in the acoustic platform by using intensive mixing generated by acoustic waves. Besides, these micromixers addressed a common challenge in the microfluidic platforms by preventing clogging of microchannels and large aggregates formation (Tabrizian, 2019). On the other hand, passive micromixers do not require any external source of energy and rely on fluid pumping energy to provide the mixing process. Obviously, they are easier to fabricate, but it's necessary to keep in mind that the shapes and designs of the micromixer is an important factor in their mixing efficiency (Tripathi, Patowari, & Pati, 2023). Most of the micromixers for liposome production are passive micromixers (Basuray, 2011; Joshi et al., 2016). Also, passive micromixers show suitable results in reproducibility and controlling liposome size.

1.3.1.2 Molecular diffusion-based micromixers

In molecular diffusion-based micromixers, Brownian motion is the reason for particle movements from a region with higher concentrations to the one with lower concentrations. In the following, three types of molecular diffusion-based micromixer are described.

1) Microfluidic hydrodynamic focusing mixer (MHF) is a micromixer that produces liposomes under continuous flow conditions. High monodispersed liposome populations would be produced using MHF micromixers with adjustable diameters by changing the ratio of aqueous to organic solvent volumetric flow rates (FRR) (Michelon, Oliveira, de Figueiredo Furtado, Gaziola de la Torre, & Cunha, 2017).

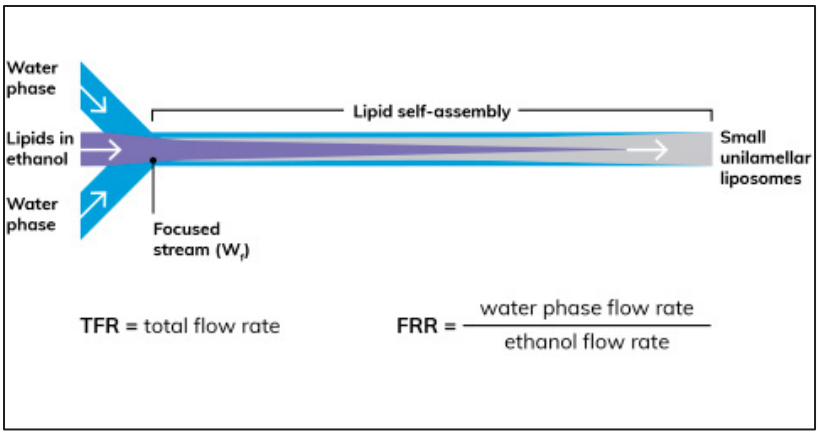


Figure 1.2 Flow focusing production method, each of the microchannels contains laminar flow, where co-diffusion of organic solvents with aqueous streams produces regions with low organic solvent concentration promoting self-assembly
Taken from (dolomit, 2020)

In Figure 1.2, the definitions of TFR and FRR are described. As previously indicated, FRR plays an important role in the control of liposome size, whereas the (Total Flow Rate) TFR does not affect the size (Joshi et al., 2016). FRR and the size of the liposomes have an inverse relationship. Therefore, by increasing the FRR, the mixing rate inside the channel increases and the liposome size will decrease. On the other hand, at the same FRR condition, the microfluidic device aspect ratio is directly proportionate to the liposome size (Michelon et al., 2017). As a result, the size of liposomes could be easily tuned by controlling FRR values in MHF mixers to obtain monodispersed populations of liposomes with no need for further homogenization steps.

2) Three-dimensional microfluidic hydrodynamic focusing (3D-MHF) micromixers were invented to increase mixing performance compared with the conventional MHF micromixers. As it's shown in Figure 1.3, these types of micromixers increase the uniformity of liposomes by increasing the flow focused area and consequently improving diffusion inside the microchannel (Hood & DeVoe, 2015; Zhang & Sun, 2021)

3) Vertical flow-focusing micromixers (VFF) opened a novel opportunity to produce scalable, stable, and higher concentrations of final liposomes in a rapid production for real-time operation. In the VFF approach, there is a stack of wide and shallow channels located in a vertical path that has been created using a multilayer thermoplastic fabrication process (Hood & DeVoe, 2015; Michelon et al., 2017).

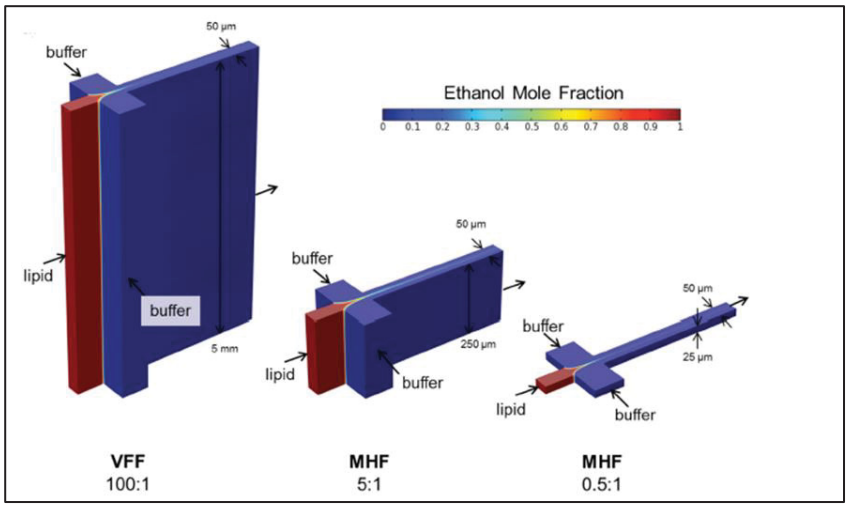


Figure 1.3 A schematic model of structure, and ethanol concentration profiles inside VFF, 3-D MHF and MHF systems and decreasing aspect ratio of microchannel from 100:1 to 0.5:1 laminar flow and uniform mixing are characteristics of all these devices
Taken from R. R. Hood and DeVoe (2015), p.5792

As shown in Figure 1.3, VFF micromixers use high aspect ratios of 100:1 to address problems of liposome production yield and produce liposomes with lower PDI. It was investigated that the production rate of VFF is about 95 mg/h which is much higher than MHF systems and improve liposomes yield nearly two orders of magnitude in comparison with MHF mixers (Hood & DeVoe, 2015).

As a conclusion, liposomes produced by MHF enhance low PDI, and suitable controlling over the liposome diameter through changing FRR with laminar flow inside the channels. Besides, by increasing the aspect-ratios of MHF, liposomes with lower polydispersity and higher production rates could be obtained. Despite all the advantages of MHF, liposome yield and liposome production rate were not suitable for industrial scale.

1.3.1.3 Chaotic advection-based micromixers

The structure of chaotic advection micromixers differed from that of molecular diffusion-based micromixers by containing some obstacles inside the mixing channel to produce flow patterns and having two inlets instead of three inlets. This type of micromixer is described further in the following paragraphs.

The function of the structures inside the mixing channel is to induce rotational flow which wraps the fluids onto each other and produces chaotic flow. The chaotic flow inside the mixing channel generates rapid mixing of lipid solution and increases the polarity and mixing efficiency (ME) (Joshi et al., 2016).

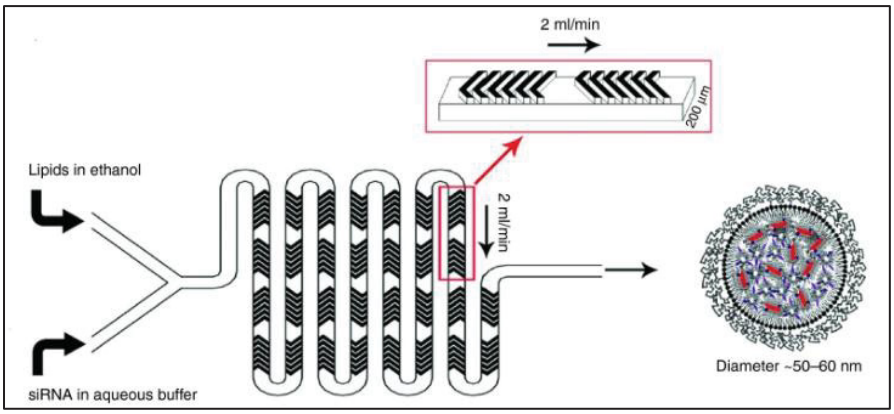


Figure 1.4 A staggered herringbone micromixer
Taken from Hyun B Choi et al. (2013)

Figure 1.4 is showing a Staggered herringbone micromixer with pattern of grooves inside the microchannel providing very rapid mixing of lipid solution in mixing channel for liposome production. In the this mixer, TFR is an important factor in controlling liposome diameter as opposed to MHF mixers. Lower amounts of FRR are necessary for higher rates of liposome production. Some advantages of using SHM micromixers include production of limited size

liposomes, production of the smallest possible liposome particles (in the range of 20 nm), and remaining only 1% of leftovers using online purification through a tangential flow filtration (TFF) device (Belliveau et al., 2012). But still, there are some disadvantages of using SHM in liposome production such as the formation of clogging, laborious fabrication due to 3D features, and not always resulting in monomodal liposome populations, which need further investigation and experiments.

In conclusion, micromixers including the SHM are widely used for production in drug delivery systems, while MHF micromixers are used to synthesis for gene delivery applications based on their different mixing principles. Despite these developments in microfluidic devices, the need for mass production in liposomal applications has not been answered yet.

1.3.1.4 Dean forces-based micromixers

Molecular diffusion and chaotic advection micromixers have limitations such as liposome yield and poor device reliability. Dean forces-based micromixers such as Periodic disturbance micromixer (PDM), by having curvilinear paths that induce centripetal forces, speed up the mixing process to answer these problems is a micromixer. Figure 1.5 shows a Periodic disturbance micromixer with a mixing channel consisting of 90 semicircular structures.

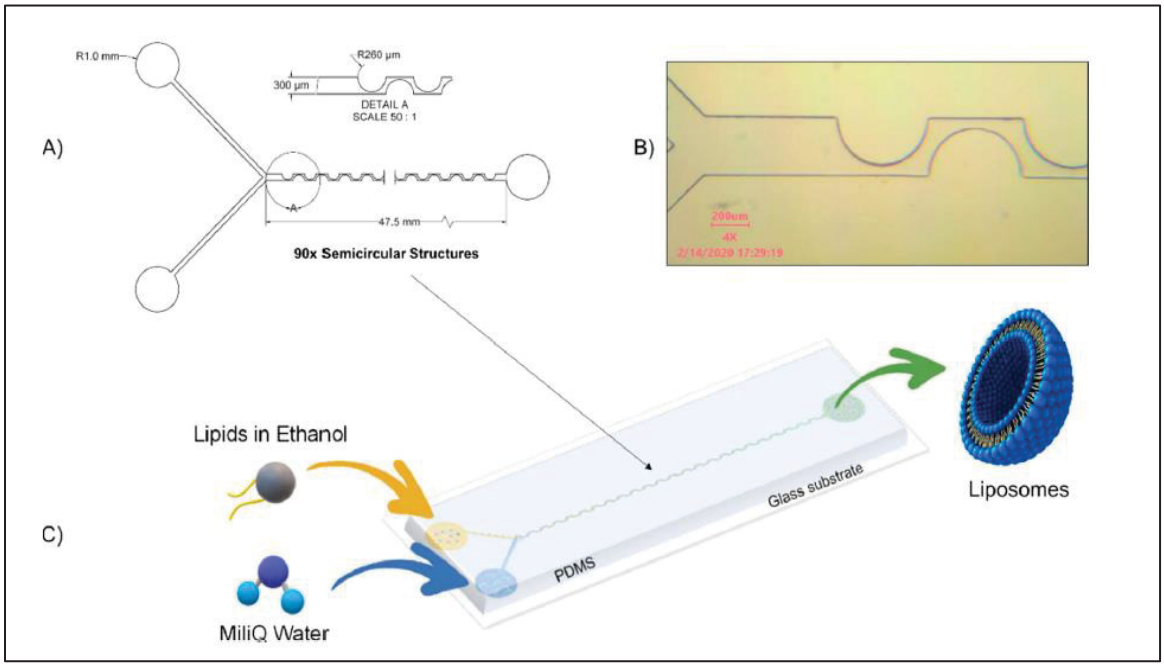


Figure 1.5 Periodic disturbance micromixer scheme A) Microchannel dimensions of the PDM, B) Enlarged photo of the microchannel with a curvilinear structure to produce Taylor dispersion and chaotic advection to speed up with the mixing process,
C) Liposome preparation process; each ethanol solvent and aqueous solvent is injected in one inlet and reaching a mixing channel with 90 semicircular structures, which increase mixing fluids from the two inlets. In the end, the final product could be collected at the outlet
Taken from R. R. Lopez et al. (2020), p.3

Overall, liposome size has been shown to be controlled by altering TFR and FRR. Multiple micromixer designs using Dean forces for producing lipid-based nanoparticles showed an increase in the yield by decreasing FRR (R. R. Lopez et al., 2020).

1.3.2 Milli mixers in liposome production

Despite microfluidic-based mixers that have limitations such as high cost for manufacturing and low production rates, milli mixers take advantage of higher production rates, better controllability over variables to tune particle's properties, and cost-efficient fabrication due to their larger dimensions and ease of fabrication. Milli mixers are also useful in the mass production of liposomes in pharmaceutical applications by having compatible characteristics

with medicinal liposomal formulations (size<100 nm; dispersity < 0.2) (Yanar et al., 2020). Flow rates can influence the ME in milli mixers in the way that by increasing the TFR, ME increased due to stronger secondary flows, and by increasing the FRR at a constant TFR, ME increased. By controlling these features, liposomes suitable for pharmaceutical applications would be obtained (R. R. Lopez et al., 2020; Yanar et al., 2020). In Figure 1.6, the structure of a milli mixer is investigated with two curved shape inlets.

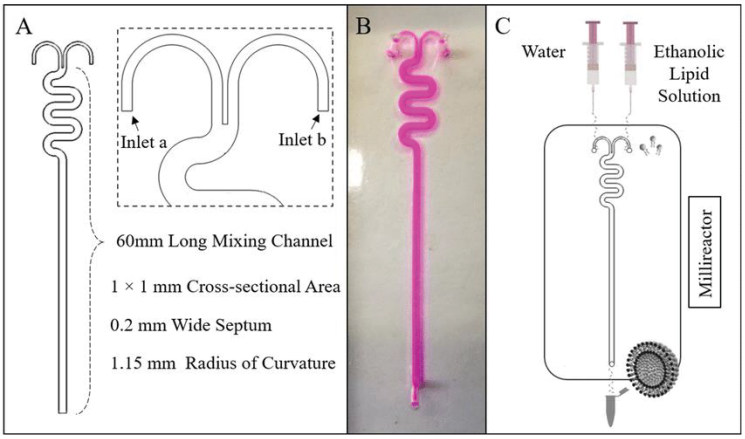


Figure 1.6 Milli mixer's structure with geometries; two curved inlets with rectangular cross-sections are separated by a septum to generate parallel flow before reach together, B) a top view of produced milli mixers, and (C) a schematic description of liposome production Taken from (Yanar et al., 2020)

In another study, Zizzari et al. have indicated that by increasing the channel volume in MHF and MHF large channels (MHF-LC) and promoting monodispersity in radial flow, milli mixers could be built with a very cost-effective and straightforward strategy (Zizzari et al., 2021). In this way, coaxial milli mixers and T-shaped milli mixers have been built using thermal bonding to assemble pre-cut glass blocks to produce the device with lower cost. Coaxial milli mixers have shown 15-20 times higher production rates comparing with MHF micromixers, and highly monodispersed liposome with liposome size suitable for pharmaceutical applications (Zizzari et al., 2021). Figure 1.7 shows the steps of production of MHF coaxial milli mixers. In pictures a and b, black arrows point to the location of the edge of the injection tube based on the T-junction area in T-MHF-LC and Co-MHF-LC structures.

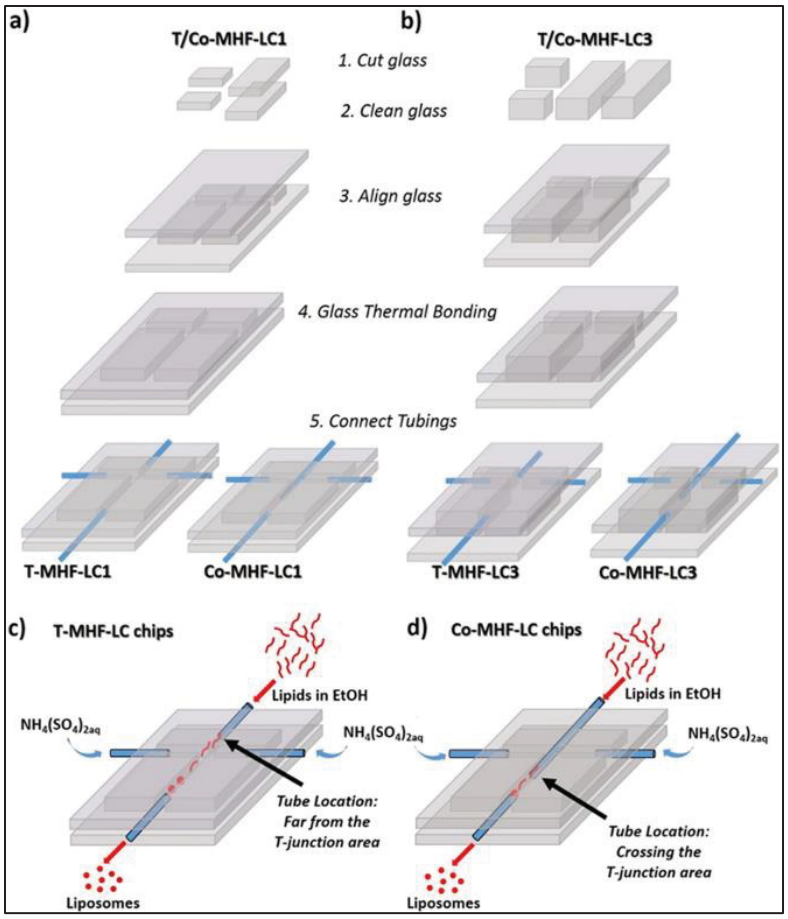


Figure 1.7 Schematic representation of structure design in milli mixers, Taken from Zizzari et al. (2021), p.4

Overall, liposomes with uniform morphology with concentrations and dimensions suitable for pharmacological applications can be produced by milli mixers. Further studies are required to investigate the influence of different structure topologies and flow rate conditions on drug loading using milli mixers.

1.4 Encapsulation of biomolecules

Liposome nanoparticles with a hollow structure in the middle of their sphere-shaped structure, can encapsulate a wide variety of biomolecules. Encapsulation of components can promote applications of liposomes, including their use as a nanocarrier for encapsulating agents for food

ingredients, cosmeceutical applications, drug delivery systems, and gene delivery applications (R. R. Lopez et al., 2020; Maeki et al., 2018).

In food ingredients, liposome encapsulation prevents taste distortions of the components in comparison with using these components in their free form (Khorasani, Danaei, & Mozafari, 2018). Liposomes are considered as suitable carriers in cosmeceutical applications, because they can act as transdermal delivery systems due to their lipid components, and improve skin hydration by their hydrophilic and hydrophobic contents (Van Tran, Moon, & Lee, 2019).

The drug's dosage is limited for administration in the body because of toxicities related to the size of the drug molecule. Liposome encapsulation prevents the accumulation of the drug and reduces its relevant toxicities and passive targeting. Also, in therapeutic applications, liposomes can encapsulate small interfering ribonucleic acid (siRNA) (Belliveau et al., 2012; López, 2020).

Generally, there are two methods for drug loading in liposomes:

1) Active loading: in this method the drug would be loaded after liposome generation.
2) Passive loading: in which the drug would be encapsulated in liposomes during liposome formation by adding it into the appropriate phase. The continuous flow in fluidic devices allows the combining the vesicle formation with liposome encapsulation (Chen et al., 2019; Joshi et al., 2016).

In passive drug loading, poorly soluble drugs (propofol) were dissolved directly in the organic solvent, then the organic solvent was mixed with an aqueous phase in a fluidic device, and as a result, drug encapsulated liposome nanoparticles were obtained. The drug's encapsulation efficiency (EE) is influenced by various liposome properties, such as their aqueous volume or rigidity of the membrane. Additionally, the nature of the drugs, which can be either hydrophobic or hydrophilic and their ability to make interaction with the membrane bilayer also impact the encapsulation process (Guimarães, 2020). The calculation of encapsulation efficiency is based on equation (1.1):

$$EE\% = 1 - \left(\frac{C_{free}}{C_{total}}\right) \quad (1.1)$$

Where C_{free} represents the concentration of free drug, and C_{total} represents the total drug concentration (Costa, Xu, & Burgess, 2014) The EE for these drugs using fluidic methods is above 50%, whereas it has been measured for conventional hydration methods to be around 20% (Kastner, Verma, Lowry, & Perrie, 2015).

Later another group investigated co-encapsulation of the components using microfluidics in the production of liposome, which starts by dissolving lipids and lipophilic drugs (e.g., glipizide) in an organic solvent and dissolving water-soluble drugs (e.g., metformin) in an aqueous phase, then mixing these two phases together. As a result, liposomes containing two types of drugs were generated. Co-encapsulation of two drugs has no influence on the liposome loading capacity in comparison with the loading of the drugs individually. Also, co-encapsulation of drugs increases the rate of drug release, which suggests an occurrence of synergistic or interference effects (Joshi et al., 2016; Zizzari et al., 2021).

Using size reduction methods after the production of large vesicles results in decreasing encapsulation efficiencies and lack of industrial scalability. (Joshi et al., 2016). There are other parameters that require to be optimized due to their impact on the encapsulation efficiency such as the mixing rate of the aqueous and solvent buffer and total flow rate (TFR).

Overall, encapsulation of the components using fluidic devices can increase the encapsulation efficiency. Further investigations are needed to decrease the rate of drug release from liposomes and increase encapsulation efficiencies to produce well-encapsulated liposomes for medicinal and industrial applications.

1.4 Increasing liposome throughput

Increasing liposome throughput is one of the most important factors in liposome production since the first time when scientists understood the applications of these spherical nanocarriers. High liposome throughput is required in industrial uses, such as in pharmaceutical and medicinal applications. Therefore, some strategies have been proposed to further increase liposome throughput described in detail below.

Compared to conventional bulk methods, continuous-flow microfluidics are used to scale and increase liposome production by controlling the physical properties of the end product to produce liposomes with characteristics comparable to industrial applications, especially in the terms of their size, lamellarity, and size distribution (Chen et al., 2019; Joshi et al., 2016).

First, nanoprecipitation and final liposome yield could be controlled in fluidic devices by providing rapid mixing. Fluidic devices can decrease complexities of nanoparticle production, such as controlling synthesis throughput, size of nanoparticles, nanoprecipitation, and the problem with the clogging of channels. As an example, sharp edges and oscillatory bubbles embedded in the microchannel of acoustic micromixers, make vigorous vortical fluid motions from acoustic energy transformation that generate rapid mixing inside the microchannels. Higher throughput of liposomes could be obtained using rapid mixing and strong vortices that prevent aggregation of nanoparticles, compared to the hydrodynamic flow focusing method (Tabrizian, 2019).

In microchannels of some of the microfluidic devices such as PDM micromixer designed by Lopez et al., fluid mixing occurs by Dean vortices that produce three-dimensional lamination by non-stop splitting and redirecting fluid stream, generating the highest mixing efficiency (López, 2020). The smallest size and uniform size distribution of lipid vesicles are outcomes made from shear forces. As a result, high throughput monodispersed lipid vesicles generated from the 3D laminating effect along with high shear stress inside the microchannel (R. R. Lopez et al., 2020).

Second, the microfluidic device aspect ratio and consequently its volumetric flow rate should be adjusted. Conventional MHF devices suffer from limited throughput because of small channel dimensions. To overcome this limitation, the aspect ratio of the channels was increased, and Vertical Flow Focusing (VFF) devices were exploited. Increasing aspect-ratio of the microfluidic device leads to an increase in the volumetric flow rates and the production rate at the same focusing conditions. In fact, by increasing FRR and TFR mixing efficiencies increased due to stronger secondary flows. And finally, higher throughput and lower PDI of the final product were obtained (Michelon et al., 2017; Yanar et al., 2020; Zizzari et al., 2021).

In general, "world-to-chip" interfacing entails fluidic interconnects, which enable the transportation of fluids from the macro-world to the micro-device. The Vertical Flow Focusing (VFF) platform takes advantage of improving the “world-to-chip” interfacing which presently constrains maximum flow rates and employing higher aspect ratio focusing channels. The exceptionally high liposome synthesis rates suggest that the automated and continuous flow in the VFF platform offers significant benefits for the large-scale production of liposomal nanoparticles for applications in drug delivery and beyond. A maximum liposome production throughput of 95 mg/h for the VFF device was demonstrated using moderate values of initial lipid concentration along with linear flow velocity (Hood & DeVoe, 2015; Maeki et al., 2018). Besides, liposome PDI and its diameter remain nearly constant for flow velocities between 10 and 30 cm/s shows that in higher flow rates, higher throughput for the VFF system can be achieved without any influence on liposome size distributions (Hood & DeVoe, 2015).

Third, operating multiple devices in parallel has a strong influence on liposome throughput. Parallelization of multiple devices is applicable in milli mixers or SHM micromixers. By parallelizing 6 SHM micromixers with TFR=72 mL/min, 580mg/h lipids would be produced, nearly 6 times more than VFF micromixer with 95mg/h yield (Belliveau et al., 2012).

In conclusion, three efficient techniques have been proposed in this section for increasing liposome production. In this study, we took Lopez’s PDM micromixer as we already have the

results of the characterization of the liposomes synthesized using this micromixer and increase the cross-section dimensions of the design for millimeter dimension instead of parallelization 10 micromixers to increase liposomes production in a cost and time effective way. This way, we aim to obtain higher liposome throughput for pharmaceutical applications to enhance liposomes as a suitable drug carrier all around the world.

1.5 Production of pure liposomes with reduced toxicity

Liposomes are composed of natural phospholipids that are biologically inert and feebly immunogenic, and they have low inherent toxicity. During liposome production, some substances such as organic solvents are used to produce lipid solutions. Most of the organic solvents are found to be toxic, and their remnants can generate toxicity and make challenges in liposome applications. Therefore, new strategies in liposome production should be taken. Also, further steps are required in liposome production in order to prevent toxicity and produce pure liposomes with reduced toxicity (Akbarzadeh et al., 2013).

In the first step of liposome preparation, chloroform is generally used for diluting reagents. Chloroform is proved to be highly toxic and low remanent of this substance may result in toxicity of the product. Therefore, the removal of chloroform should be accomplished by leaving the mixture in an atmosphere free of oxygen. A nitrogen stream would be useful to prevent lipid degradation and speed up the process. Then, vacuuming of the lipid mixture should be done for 24 h in order to remove the solvent totally (Liu et al., 2019; R. R. Lopez et al., 2020).

Some of the organic solvents that can be used in liposome production are ethanol, IPA, methanol, and Transcutol®. Methanol and IPA proved to be toxic, and it is better not to use these substances as an organic solvent. Most of the organic solvents are toxic and need further steps of final product purification. Transcutol® has some contaminations that make it toxic, but it is proved to be safe in human usage if it is highly purified (over 99.9%) (Osborne & Musakhanian, 2018). Also, it can avoid filtration steps for removing the solvent and helps in drug delivery as opposed to IPA and methanol. In the case of using organic solvents other than

Transcutol®, to remove their residues from the vesicles, extra purification steps, such as dialysis, are required. In the dialysis process, before starting the process, dialysis tubing should be soaked for 2 h in water. This process should be performed using PBS at PH=7.4 to remove the solvent from liposomes (Joshi et al., 2016).

In SHM micromixers, online purification and characterization left 1% of drug leftovers. In this technique, the SHM micromixer directly connects to a tangential flow filtration (TFF) device. This device works as an integrated solvent removal during the liposome formation process. As a result, only 1% of the non-incorporated drug is left in liposomes (Dimov, Kastner, Hussain, Perrie, & Szita, 2017).

Overall, removing remanent of organic solvent and non-incorporated drugs in liposome production using micromixers is still challenging and needs further studies and investigations to produce pure liposomes suitable for drug delivery applications.

1.6 Discussion

In this part, different methodologies for liposome production using micromixers and milli mixers are provided in Table 1.2 to compare these methodologies and propose a solution to produce liposomes in higher rates and increase the production yield with reduced toxicity.

Table 1.2 A comparison on different methods of liposome production

Working principle	Devices' name	Improvement(s)	Advantage(s)	Disadvantage(s)
Molecular diffusion-based micromixer (Joshi et al., 2016)	MHF	Continuous flow condition	1) Monodispersed liposome 2) Adjustable diameters	- Low liposome yield - Low production rate
Molecular diffusion-based micromixer (Dimov, Kastner, Hussain, Perrie, & Szita, 2017)	3-DMHF	Increasing mixing performance	1) Improving diffusion 2) Increasing liposome uniformity	- Low liposome yield - Low production rate
Molecular diffusion-based micromixer (Hood & DeVoe, 2015)	VFF	1) Increasing aspect ratios 2) Rapid mixing	1) Increasing liposome production yield 2) Decreasing PDI	-Low production rate
Chaotic advection-based micromixer (Hood & DeVoe, 2015)	SHM	1) Using herringbone structures 2) Increasing the polarity 3) Increasing the ME	1) Ease of design 2) Production of the smallest liposomes 3) Online purification	-Clogging formation - Laborious production -Not always leading to monodispersity
Dean forces-based micromixer (R. R. Lopez et al., 2020)	PDM	1) Using curvilinear mixing channel 2) Inducing centripetal forces & higher mixing	1) Increasing device reliability 2) Improving liposome yield	- Costly to produce - New design and still needs studies
Milli mixers (R. R. Lopez et al., 2020; Mahsa Sedighi1 & Jörg Huwyler2 & Dominik Witzigmann2, 2018)	Milli mixers/Coaxial milli mixers	1) Increasing aspect ratios to millimeter scale 2) Better tunning particle's properties 3) Speeding up the mixing 4) rectangular profile	1) Very cost-effective and straightforward strategy 2) 15-20 times higher production rates 3) Highly monodispersed liposome	- Rectangular cross-sections may increase the risk of clogging because of the edges

According to previous studies, and as the Table 1.2 shows, continuous flow is essential for liposome production with lower PDI. Besides that, increasing aspect ratios of the mixers can help with improving liposome production rate and liposome throughput (Hood & DeVoe, 2015). Production of liposomes in a cost-effective way can be obtained by using milli mixers, which are easier to fabricate because of their higher aspect ratios (Yanar et al., 2020). Zizzary et al. have produced milli mixers with rectangular cross-section by increasing the channel volume in MHF and LC-MHF, and promoting monodispersity in radial flow (Zizzari et al.,

2021). Still, there is a need for a novel strategy that can increase liposome production to industrial-scale production and decrease its toxicity.

A milli mixer should be produced that takes advantage of acoustic energy to produce higher amounts of liposomes. The structure of the milli mixer should contain two curved shape inlets that have a septum in between to produce a laminar flow regime and uniform mixing of the reagents. The cross-section structure of these milli mixers would better be circular. Milli mixers with a rectangular cross-section may increase the risk of clogging, because the forces produce from the walls of the channels to the fluid inside the channel (centripetal forces), are different in the edges of the rectangular structure in comparison with its sides. But these forces are dispersed equally in channels with a circular cross-section. Therefore, the preferred structure for the proposed milli mixers would be microchannels with circular cross-sections.

Parallelization of the proposed cost-effective milli mixers can be an alternative to increase the production throughput more than before to achieve mass production for use on an industrial scale. Also, further studies are required to find other designs of milli mixers with more appropriate properties, which can increase the production yield while maintaining liposome psychochemical characteristics.

1.7 Chapter 1 conclusions

In this chapter, different strategies for producing liposomes were presented. The main focus was on fluidic devices, which proved to be more effective than conventional methods. Some of these devices are based on microfluidics, which are mainly based on molecular diffusion, chaotic advection, or Dean-forces, to produce size-controlled liposomes at higher rates. Others are based on milli mixers, which are used to increase liposome throughput while maintaining mixing efficiency inside the mixing channel simultaneously.

We have proposed a solution for problems with production, scaling, production costs of mixers and maintaining physicochemical characteristics of the final liposomes by controlling over

mixing efficiency in different points of the channels. The new design might be helpful to improve the production rate, increase production of liposomes and reduce the costs related to producing multiple fluidic mixers for parallelizing and increasing production rate. In order to control over physiochemical characteristics of liposomes including size, PDI, and other influential characteristics in a scaled-up design, altering variables such as FRR, TFR, and PBS is essential. Due to the larger dimensions of the mixing channel, it is possible to obtain liposomes with higher size and PDI due to the possible changes in fluid dynamics and mixing efficiency of the mixing channel. Future studies could focus on optimizing milli mixers design along with an external source of energy (active milli mixers) which can increase liposome throughput and control liposomes to produce monodispersed populations in a more cost-effective way and make the final product suitable for industrial applications to reach these carriers to the world.

CHAPTER 2

MATERIALS AND METHODS

This chapter will provide a comprehensive account of the techniques employed to synthesize liposomes in this work and the experimentation determining physicochemical parameters. In addition, the characterization process of the final product utilized in various experiments presented within this thesis will be thoroughly elucidated.

2.1 Milli mixers and micromixers

Milli mixers and micromixers are designed and fabricated to facilitate efficient mixing in producing lipid-based nanoparticles. Microfluidic mixers or micromixers are generally designed with channel dimensions ranging from tens to hundreds of micrometers. Different mechanisms are used in these devices including laminar flow, chaotic advection, diffusion, and turbulence. Micromixers are usually fabricated using different techniques such as photolithography, soft lithography, and sometimes 3-D printing using materials such as Polydimethylsiloxane (PDMS), glass, resin, and silicon (Cai et al., 2017).

As micromixers are prone to clogging, more expensive and less durable because of their delicate structures, for larger volumes, milli mixers can provide cost-effective solutions (Zizzari et al., 2021). Milli mixers are a suitable option for pharmaceutical manufacturing and industrial chemical processes because they operate on a larger scale with millimeter-range channels. They employ similar mixing principles but require design simulations and imaging to control efficiency at larger scales. In the following subsection we further discussed the design and fabrication of milli mixers. In Table 2.1, micro and milli mixers are compared based on different aspects.

Table 2.1 Comparison of micro and milli mixers

Aspect	Micromixers	Milli mixers	References
Scale	based on a smaller scale (microliters and nanoliters)	based on a larger scale (milliliters)	(Dong, Wen, Zhao, Kuhn, & Noël, 2021; Hessel, Löwe, & Schönfeld, 2005)
Precision	higher precision because of smaller scale	lower precision compared to micro mixers	(Dong et al., 2021; Hessel, Löwe, & Schönfeld, 2005)
Applications	Used for microfluidics, lab-on-a-chip devices, chemical reactions requiring precise control	More suitable for laboratory, industrial and high throughput processes	(Dong et al., 2021; Elvira, i Solvas, Wootton, & deMello, 2013)
Scale of synthesis	Miniaturized chemical synthesis	Large-scale chemical reactions	(Gobert, Kuhn, Braeken, & Thomassen, 2017)

2.1.1 Milli mixer design and fabrication

To study liposome formation using milli mixers, we designed and fabricated different types of milli mixers such as PDM, Y-shaped and Serpentine. These milli mixers are scaled-up versions of existing micromixers to ensure that the results are comparable. Each one of the milli mixers has two inlets on one side and an outlet on the other side which are connected to each other by channels inside the structure. The two inlets meet each other with a Y shape design and connect to a mixing channel where the synthesis of liposomes happens and eventually the mixing channel connects to the outlet. A stable flow profile is ensured using this design at the inlet channels (López, 2020).

To investigate and compare liposome formation in a milli mixers vs a micromixer, we scaled up and designed three different milli mixers with increasing cross-sectional dimensions using SolidWorks 2023 and simulate all the designs using COMSOL Multiphysics® 6.1 to evaluate the structures before printing them for liposome synthesis. All the millimeter mixers are designed and scaled up based on an existing micrometer mixer. In this work, we obtained the designs of the Y-shaped, serpentine and PDM micrometers mixer from Karthikeyan et al., Tripathi e al., and Lopez et al. respectively, and in order to achieve millimeter-scale channels we scaled up the dimensions using SolidWorks by a factor of 3, 5, 10 and 15 in order to investigate which dimensions can increase the yield while producing liposomes with required physicochemical characteristics (Karthikeyan & Sujatha, 2019; Ruben R Lopez et al., 2021; Tripathi, Patowari, & Pati, 2023). In Figure 2.1, the scaled-up design of the PDM milli mixers is shown in the SolidWorks software.

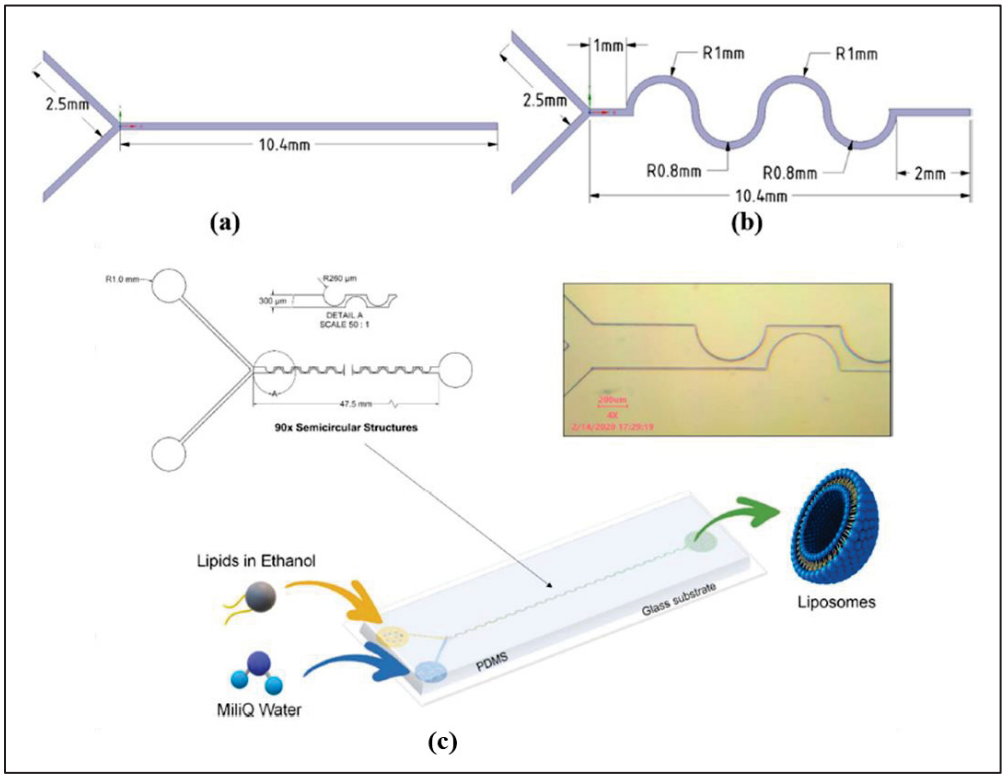


Figure 2.1 Original designs of a) Y-shaped, b) serpentine, and c) PDM micromixers Taken from (López, 2020; Tripathi, Patowari, & Pati, 2023)

In order to enlarge the cross-sectional dimensions of the micromixers and convert them to milli mixers while maintaining efficiency, it is necessary to do simulation of the milli mixers designs to determine the point of the mixing channel where efficient mixing occurs. To achieve this purpose, all milli mixers designs were uploaded to COMSOL Multiphysics® 6.1 for simulating the liquid flow and estimating the mixing efficiency of the channels. Figure 2.2 displays simulations of the PDM and Serpentine milli mixers when FRR equals 1.

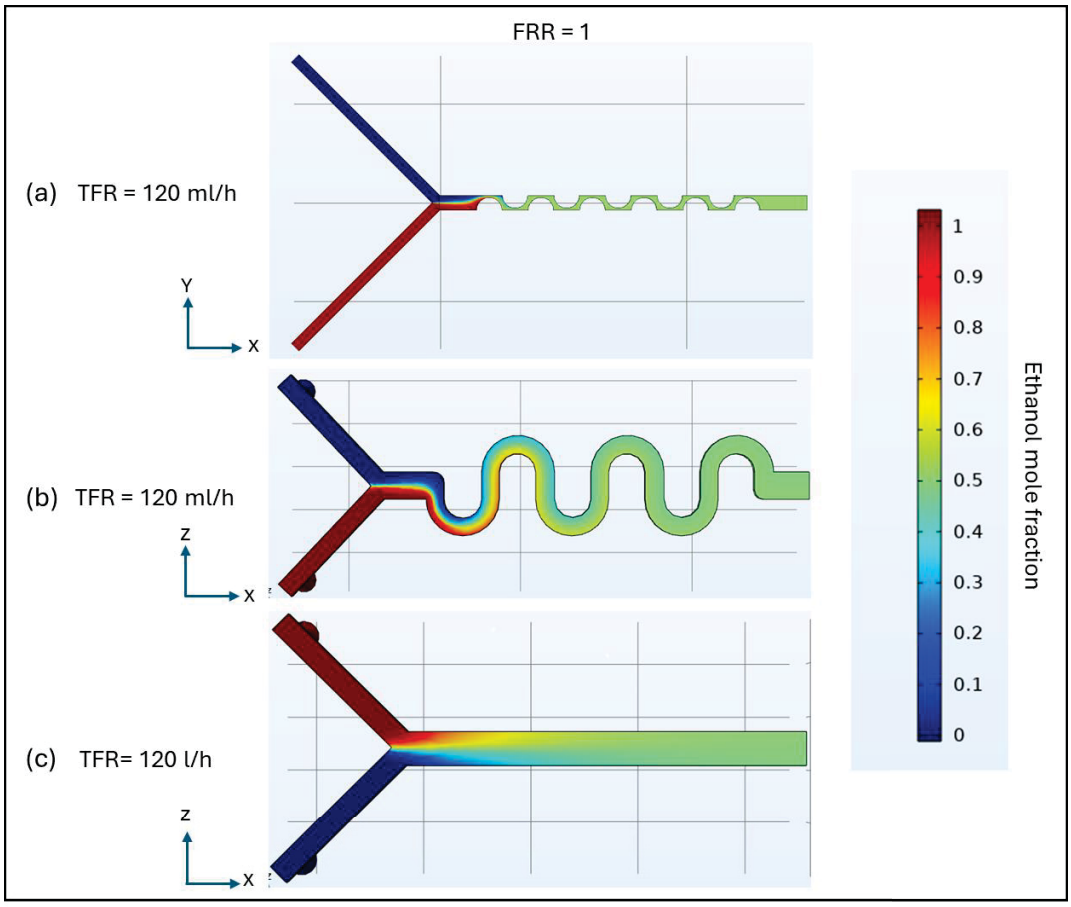


Figure 2.2 Numerical simulations of the concentration profiles in PDM and Serpentine milli mixers at FRR = 1 and a constant TFR = 120 mL/h

(**a**) Upper view of the mixing channel of the PDM milli mixer,

(**b**) Upper view of the mixing channel of the serpentine milli mixer,

(**c**) Upper view of the mixing channel of the Y-shaped milli mixer

After evaluating the design of the structure in COMSOL, in the next step, the structure must be fabricated. Usually, the micro and milli mixers are fabricated using materials such as PDMS (polydimethylsiloxane) due to its biocompatibility, transparency, and high flexibility (Waheed et al., 2016). Figure 2.3 displays a PDM milli mixer fabricated using PDMS. Recently, 3D printing is prioritized for fabricating microfluidic and milli fluidic structures instead of traditional PDMS molding because it offers faster prototyping, higher design flexibility, improvement in performance and durability, and gives the opportunity to create complex designs. It can also be more cost-effective for small-scale production.

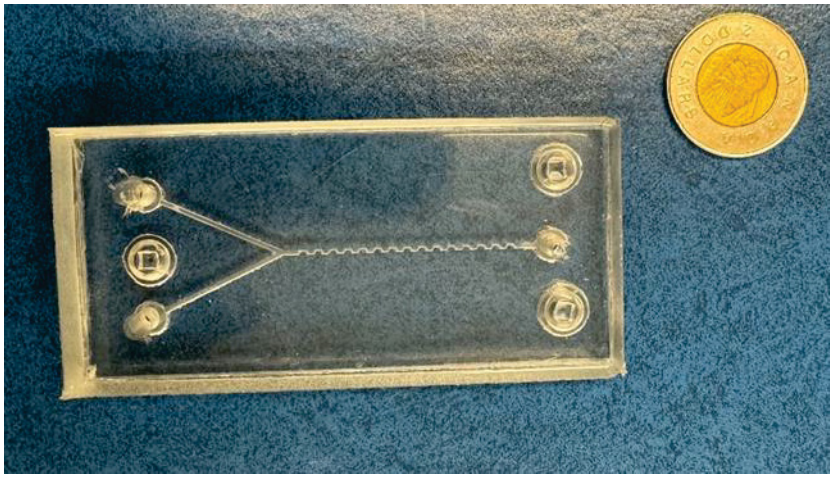


Figure 2.3 PDM milli mixer fabricated using PDMS

In our experiments, we utilized two different 3D printers from different brands. Figure 2.4 illustrates the two 3D printers used in our study. The left image displays the 3D printer from Ecole des technologie supérieure (Formlabs 3D Printer, Form 3, SLA 3D Printer), while the right image depicts the 3D printer from McGill University Health Center (MiiCraft, Prime 4K). The precision of the 3D printer is a critical factor for achieving mixers with accurate measurements. Formlabs 3D printer offers three different accuracies of 0.100mm (fastest), 0.050 mm, 0.025 mm (Finest Features), whereas the MiiCraft printer provides higher accuracy of 5 to 500 μm.

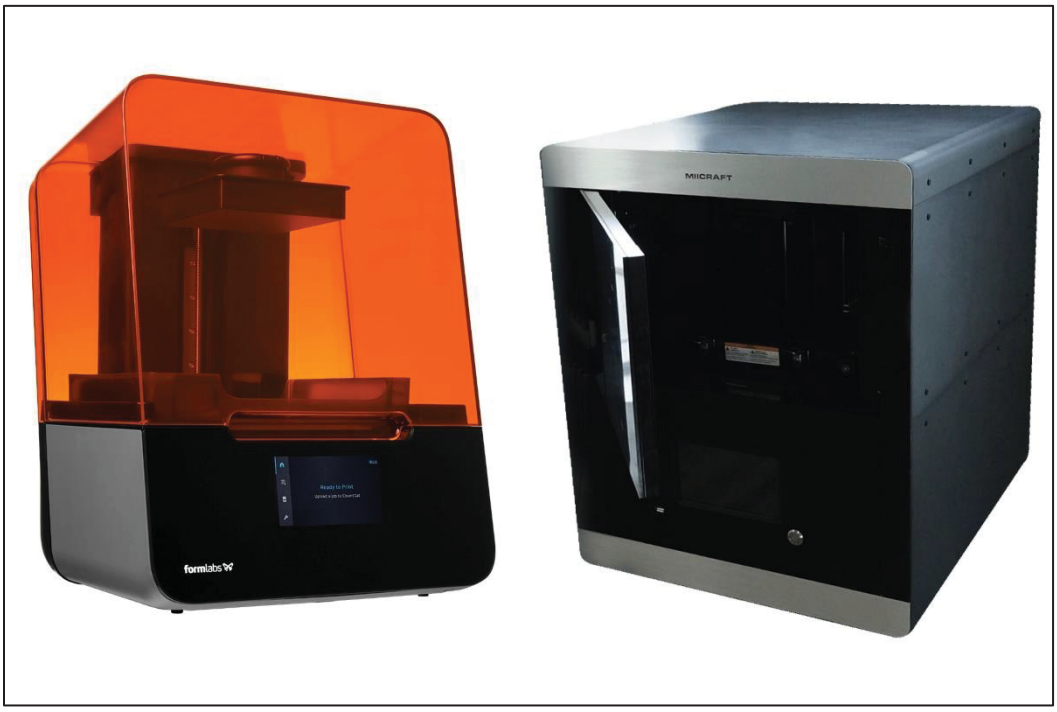


Figure 2.4 3D printer devices, the left one is Formlabs 3D Printer, Form 3 taken from (Formlabs) and the right one is MiiCraft, Prime 4K taken from (Miicraft)

We need to save our design in a format that is compatible with 3D printers such as STL (Standard Tessellation Language) on SolidWorks. The resin used in the Miicraft 3D printer is Biomed clear resin which is purchased from (CADworks3D) and amber resin purchased from (Formlabs).

Once the printing is finished, we need to carefully detach the structure from the build plate using a spatula. After removing the structure, we need to wash off the resin from the surface of it. In order to do that, we need to prepare some hot soapy water and soak the structure in it. We leave it inside the hot water for a couple of minutes then we softly rob its surface to remove residual resin from the surface.

Now it's time to clean resin from the channels inside the structure. In order to do so we need 5-10 ml of isopropanol in a syringe. We inject isopropanol using a syringe through the outlet of the channel. In the case of major clogging, we need to push with our hand a little bit to remove the extra resin caused the clogging from the channel then we continue washing with a pump. The flow in the pump should be set to 80 ml/h. In the following step, we need to put the structure inside the UV curing chambre (or form cure purchased from Formlabs) to harden the resin and achieve the hardness of the structure to be ready for synthesis. Figure 2.5 shows the result of 3D printing of the three milli mixers that we have designed in this project.

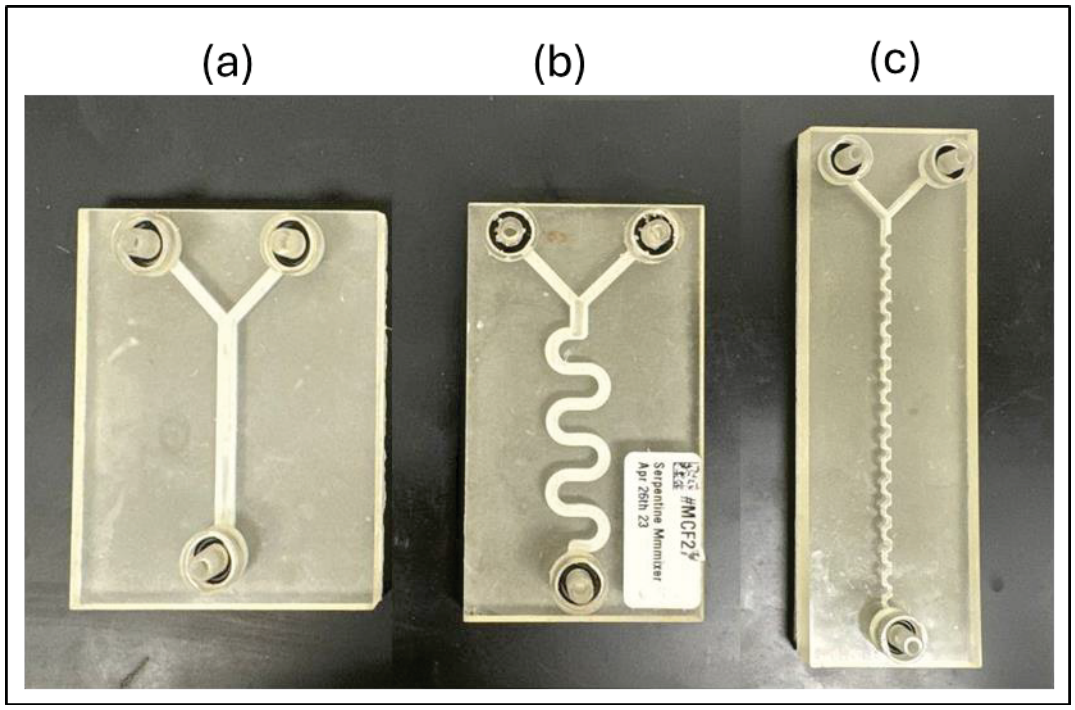


Figure 2.5 Scaled up milli mixers; (a): PDM, (b): Serpentine, (c): Y-shaped milli mixers

In Figure 2.5, we can see milli mixers printed using amber resin and Biomed clear resin. As we can see, each milli mixer has two inlets on one side and a single outlet on the other side. The inlets are configured in a Y-shaped cross and they come together leading into the mixing channel which in one case is straight (Y-shaped) and the other cases is circular or semicircular structure (PDM and Serpentine) (López, 2020).

2.1.2 Characterization and imaging of 3-D printed milli mixers

Characterizing for mixing efficiency of milli mixers prior to using them is essential to improve their functionality and the synthesize of liposomes. A digital microscope (Keyence, VHX-7100) is a key technique one can use for this purpose because it is capable to provide high-resolution imaging and flow dynamics and exact measurements of the internal structures. In Figure 2.6, the set-up of the digital microscope is depicted.

Figure 2.6 Digital microscope set-up for imaging milli mixers purchased from Keyence set-up

To achieve accurate visualization of the flow inside the milli mixers, it is necessary to utilize dyes and ensure the structure is thoroughly cleaned to prevent contamination. The device must be configured to capture high-resolution images and video recordings, which are crucial for analyzing the geometrical features of the milli mixer before starting synthesis.

2.1.3 Milli mixing setup

The liposome production setup is equipped with two syringe pumps, namely the Pump 11 Elite and Pump 11 Pico Elite (Harvard Apparatus Canada, Montreal, QC, Canada). Additionally, it includes a milli mixer and a hot plate, the EchoTherm HS40 Programmable Digital Stirring Hot Plate from Torrey Pines (115V). The milli mixers used in this experiment were Y-shaped milli mixer, serpentine milli mixer and Periodic Disturbance milli mixer (PDM). Design, simulation and fabrication of milli mixers are further discussed.

The control of the pumps and the hot plate is effectively managed using the LipoSynthesis software (Version 2.2.1), which was developed by Luz-Maria Sanchez. In Figure 2.7, there is a schematic picture of the process of production of liposomes and Figure 2.8 shows the actual fabrication set-up.

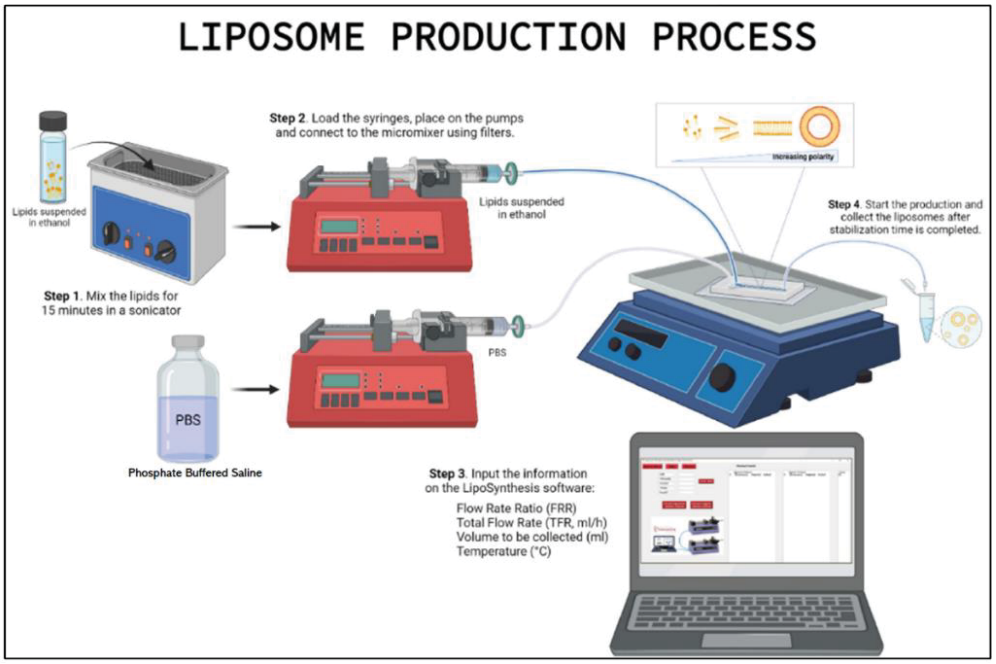


Figure 2.7 A schematic picture of the Process for liposome production showing producing liposomes step by step Taken from (Zouggari, 2022)

Figure 2.7 shows all the steps we need to take for synthesis of liposomes. According to step 1, before starting the synthesis it is necessary to mix the lipid mixture in a sonicator for 15 minutes at 45°C. It is necessary to do sonication of the lipids before synthesis for increasing homogenization and dissolution while decreasing aggregation of lipids by breaking down the aggregates (Akbarzadeh et al., 2013). This would facilitate better liposome formation. If there is no sonicator device around, it is possible to heat up the sample on a heating plate and then vortex it 3 times for 10 seconds which may be less effective.

Then in step 2, we need to load one syringe with lipid mixture which is a combination of DMPC:CHOL:DHP with molar ratios of 5:4:1 and it is described in detail in the following section, suspended in ethanol. In this project, we chose this formulation of lipid mixture because this formula was used by Lopez et al. in his project for synthesis of liposomes in PDM micromixer. In fact, we scaled up his design of the PDM micromixer to PDM milli mixer

aiming to compare the results of liposomes synthesized in larger scale (millimeter scale) vs the ones synthesized in smaller scale (micrometer scale).

We need to take another syringe and fill it with 10 mM of Phosphate Buffered Saline or PBS (pH 7.4) is our aqueous phase for synthesis of liposomes. PBS is an ionic solvent which affects final liposome physicochemical characteristics and specially zeta potential. Liposomes synthesized using PBS as aqueous phase are proved to have more negative surface charge or zeta potential which contributes to preventing aggregation of the particles and stability of colloidal systems because of the electrostatic repulsion (Zouggari, 2022). Stability positively influences the size distribution of the particles. Samples with uniform size distribution are more suitable for drug delivery systems and effective encapsulation. Besides that, negative zeta potential has an important role in reducing non-specific interactions with cells and proteins which improves biocompatibility (Bhattacharjee, 2016). The percentage of PBS is changed in this project from 4% to 12% to further investigate the effect of PBS on liposome phytochemical characteristics. Afterwards, we must place those two syringes (syringe filled with lipid mixture and the syringe filled with PBS) to the related pumps (organic pump and aqueous pump). It is essential to use filters before introducing the syringe to the structure to prevent clogging.

In the following step, which is step 3, we set the synthesis conditions on the LipoSynthesis software. The conditions such as Flow Rate Ratio (FRR) which represents the proportion between the flow rates of the aqueous and organic solvents, Total Flow Rate (TFR) which refers to the velocity of the fluid at the outlet of the milli fluidic device, the required volume of the liposomes and the temperature of the synthesis are inputs of the software.
The TFR and FRR are calculated using formula 2.1 and 2.2:

$$Q_{org} = \frac{TFR}{(1 + FRR)} \tag{2.1}$$

$$Q_{aq} = \frac{FRR * TFR}{(1 + FRR)} \tag{2.2}$$

In equation 2.1, Q_{org} represents diluted lipids' flow with the organic solvent flow (ml/h), and in the second equation Q_{aq} represents aqueous solvent flow (ml/h). Each one of the syringes will deliver the contents of a 10ml BD syringe into the milli mixer. One of the syringes contains lipids diluted in an organic solvent (ethanol in this case) which would be placed in the organic solvent, while the other one contains the polar solvent necessary for liposome formation (Phosphate or Citrate Buffer) which would be placed in the aqueous solvent pump. Both of the syringes are connected to the system via tubes (Masterflex® Chemital tube) and 0.22µm filters (Millipore Express PLUS Membrane Filter) which prevents big particles and bubbles interring the structure, and Masterflex® Chemital tubing (Zouggari, 2022).

In the last step, we initiate the pumps using the program. Within the milli fluidic device, organic and aqueous solvents are mixed under highly controlled conditions. After 90 seconds of the stabilization time, which is the time to ensure that the sample is produced under laminar flow conditions, we can collect the liposomes (R. R. Lopez et al., 2020).

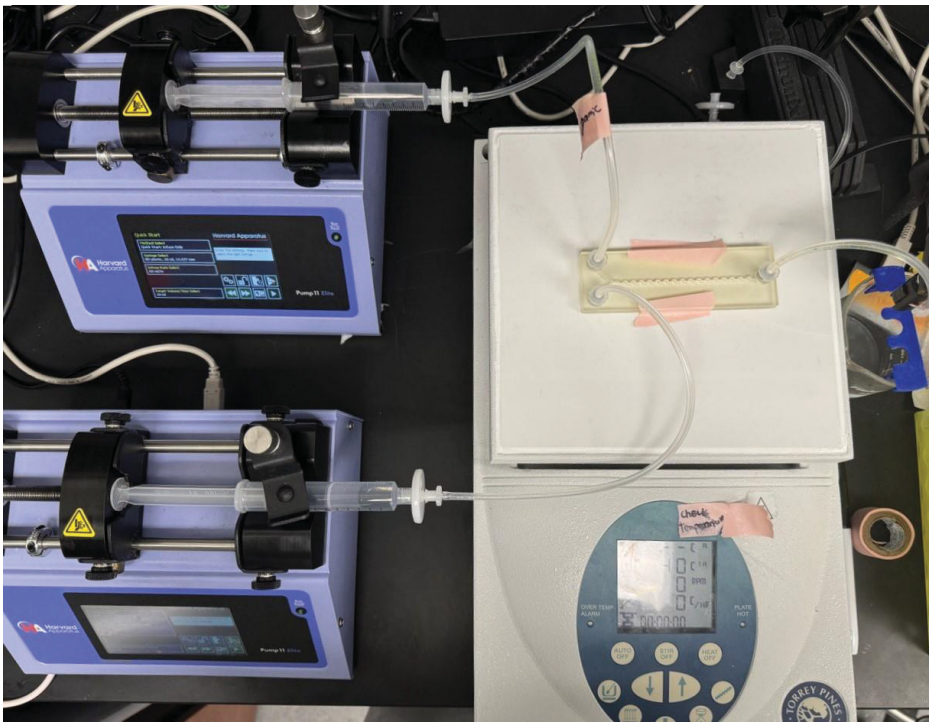


Figure 2.8 Liposome fabrication set-up in the laboratory

As displayed in Figure 2.8, on the right side of the chip there is an outlet connected to a tube which is bringing liposomes to a waste bottle where we can further dilute the liposomes to the required concentration. The aim of dilution of the liposomes when collecting them is for liposome characterization, since each instrument for characterization requires specific concentrations of liposomes. After collecting the samples in vials, they were placed in the room temperature to cool down for 15 minutes before being properly stored at 4°C.

2.2 Liposome production

From an energy point of view, the liposome formation process pursues a specific sequence. As presented by Lasic et al., lipids come together to form a disk-shaped lipid bilayer structure, where the energy is related to the perimeter of the disk due to the exposure of hydrophobic tails to the surrounding aqueous media (Lasic, 1988; Lasič, 1987). This stage allows for the addition of new lipids to the disk or the fragmentation of the structure into smaller pieces if destabilized, achieved through processes like electroformation induced by electrical fields (Dimitrov & Angelova, 1988). To minimize the energy associated with the disk's edges, the lipids close in on themselves, forming spherical vesicles. However, this bending process requires additional energy. Consequently, the overall energy of the system increases as the structures bend. Subsequently, as the vesicles close, the total energy decreases, but the liposomes remain trapped in a high-energy state (López, 2020; Patil & Jadhav, 2014). Developing precise and highly controllable synthesis methods is essential for taking advantage of the complete potential of nano particles and effectively regulating their behavior in biological environments. This includes aspects such as biocompatibility, biodistribution, interaction with the immune system, and therapeutic effectiveness (Tabrizian, 2019).

The fundamental of several nanoparticle preparation methods is based on the nanoprecipitation technique, which is also popular as the solvent displacement method (López, 2020). The control of nanoprecipitation can be achieved using milli fluidic devices that regulate the rate and amount of each solvent throughout the mixing process. In this study, the Y-shaped mixer,

Perpendicular mixer and Periodic Disturbance Mixer (PDM) in millimeter size are employed, and their detailed explanation will be presented in this chapter.

2.2.1 Lipid preparation

Lipids are vital elements of cellular membranes. They are categorized in various classifications based on structural and compositional differences and they can also demonstrate solubility in non-polar, organic solvents such as chloroform and ethanol. The lipids were purchased from Avanti Polaris Inc. and Echelon Biosciences. In this case, the lipids are in the form of a suspension in chloroform. Therefore, it is necessary to dry the sample using a technique named speed vacuum at 45°C. When the lipids are suspended in chloroform, they are suitable to use for up to one year. However, when lipids are in the powder form and we dilute it directly in ethanol, their stability is limited to two weeks to a maximum of one month.

A crucial precaution is ensuring that all materials in contact with chloroform are resistant materials, such as vials, glass pipette tips, and PTFE (purchased from Sigma Aldrich) and all procedures involving chloroform should be conducted under a biologic hood. Figure 2.9 shows chloroform resistant vials used for lipid preparation. Additionally, for disposal of chloroform waste, a specific waste bottle resistant to chloroform is needed.

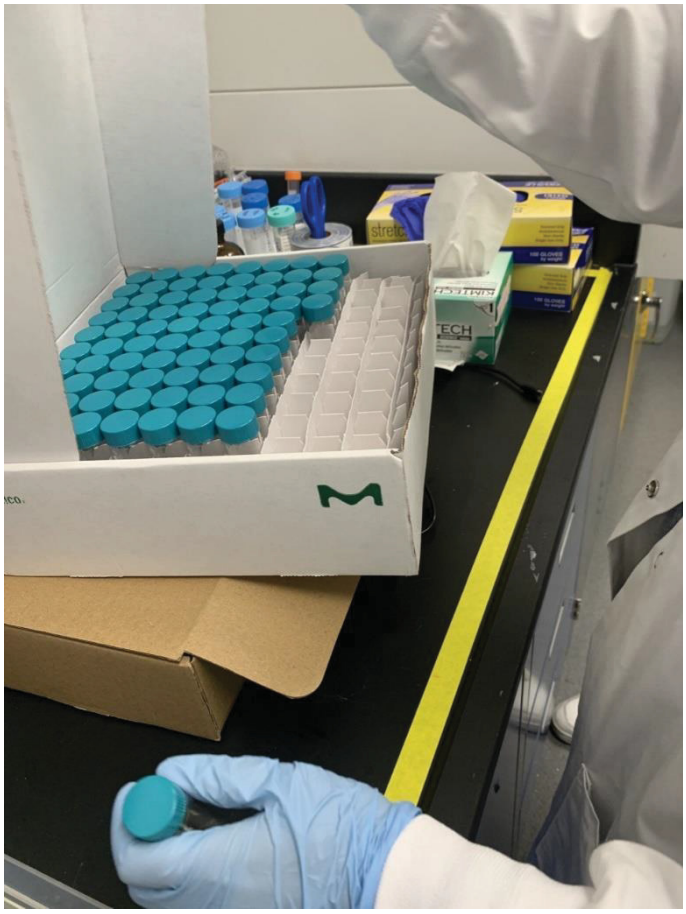
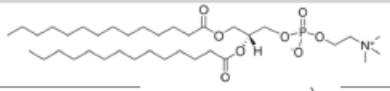
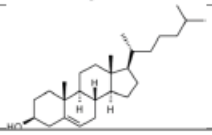
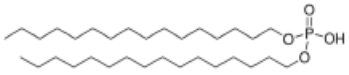


Figure 2.9 Chloroform resistant vials with PTFE caps purchased from Sigma Aldrich

Aiming to produce liposomes with diverse physicochemical characteristics, different lipid formulations have been investigated. In this work we used anionic liposomes because of their advantages in terms of increasing cellular interaction and uptake. Besides that, anionic liposomes offer great potential opportunities for targeted delivery, specifically considering the different factors affecting the cellular uptake aside from electrostatic interactions (Zouggari, 2022). Anionic liposomes, characterized by a negative Zeta Potential, were synthesized using a formula of 50% DMPC (1,2-dioleoyl-sn-glycero-3-phosphorethanolamine), 40% cholesterol, and 10% DHP (Dihexadecyl phosphate) (Zouggari, 2022). The DMPC we utilized in our formula was pre-suspended in chloroform and it came in a liquid form. Conversely, cholesterol was in powder form. Table 2.2 shows lipids used in lipid mixture in this study, their specific name and formulation.

Table 2.2 Lipids used in lipid mixture and their formulation. Retrieved from Millipore Sigma

Lipid	Name	Formula
DMPC (14:0 PC)	1,2-dimyristoyl-sn-glycero-3-phosphocholine	
CHOL	Cholesterol	
DHP	Dihexadecyl phosphate	

In order to initiate preparing the formulation, it is necessary to accurately measure the volume of the lipid mixture that we need in synthesize of lipids overall for all of the samples. The concentration of lipids should always be kept at 10 mM in lipid mixture. Therefore, we need a total concentration of 10 mM of the above-mentioned ratios of the lipids in the formulation.

Also, the exact quantity of a specific lipid required for a lipid mixture is measured using the following formula (Zouggari, 2022):

$$Mass_{lipid} = R * C_f * V_T * M_W \tag{2.3}$$

In this formula, R represents for the molar ratio (%) of the required lipid, C_f represents lipids' final concentration, V_T is a representative total volume and M_W is the molecular weight of the under-study lipids. As an example, in an anionic formulation at R=50%, the molecular weight of DMPC is M=677.93 g/mol. If we want to produce 2ml of volume with a 10 mM of lipid concentration the mass of require DMPC would be (Zouggari, 2022):

$$Mass_{DMPC}(gr) = 50\% \times 10\,mM \times 2\,mL \times 677.93\,g/mol \tag{2.4}$$

In the subsequent step, we can mix the mixture using a vortex. Following this, we employ an evaporator to eliminate residual chloroform from the lipid mixture. Before starting the evaporation process in order to prevent contamination, cleaning of the apparatus, including the flasks, and associated implements is necessary. All tools utilizing in chloroform evaporation process should be diligently cleansed and sealed with foil.

After attaching the distilling trap to the rotary evaporator, which is used to prevent sample contamination during the process, the vacuum switch is turned on to maintain the seal during evaporation. The router is regulated to 100 rpm and subsequently, the flask is submerged in a water bath with a temperature of 42°C. The liquid evaporates gradually and upon observation of white bubbles forming at the bottom of the flask, which indicates the completion of liquid evaporation, it is necessary to turn off the vacuum switch while the sample is supported with the other hand. Careful removal of both sample and waste flasks is crucial during this procedure. Due to the presence of chloroform in both flasks, they should be handled with both hands to ensure safety. Before removing the flasks from under the hood, they should be sealed with a paraffin to prevent contamination and avoid inhaling chloroform. Finally, proper disposal of waste is conducted in a designated waste bottle.

All of the flasks and tools used in this process should be placed in the desiccator. The desiccator should be closed properly, and the vacuum should turn on overnight. On the other day we can resuspend lipids inside ethanol at a 10mM concentration using ethanol spray bottle and wash the flask three times to make sure all the lipids are diluted inside the ethanol. The waste flask and other tools should be kept for one more day under vacuum and wash them using ethanol and MilliQ water. We should let the flasks dry in the chemical hood for two more days.

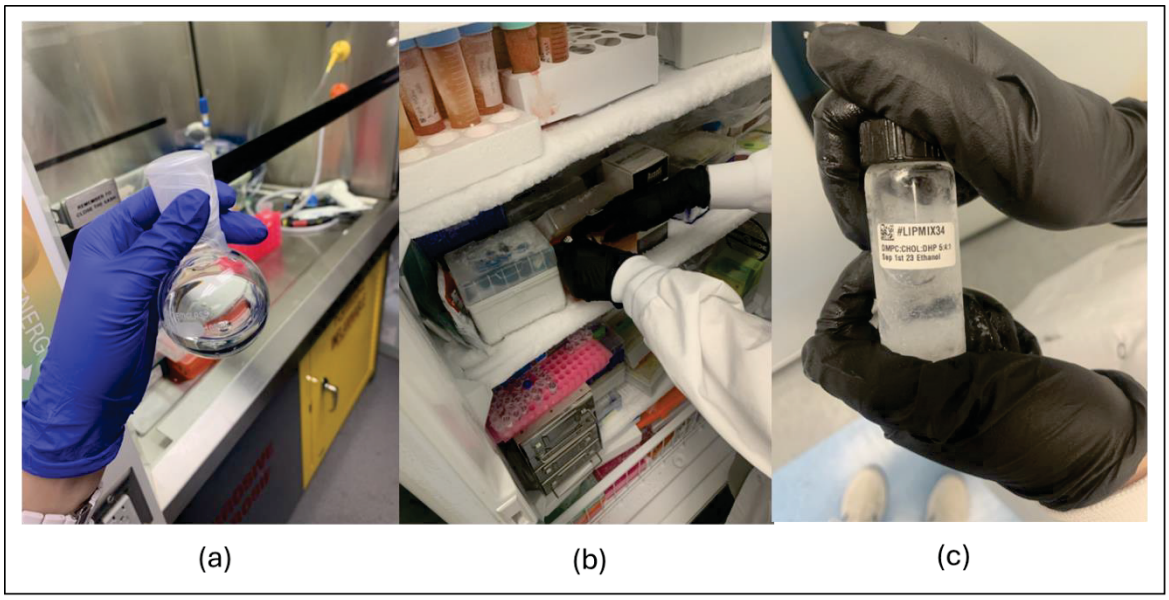


Figure 2.10 Lipid mixture storage; (a): properly sealed waste of chloroform from evaporator, (b): -20 °C refrigerator storage unit for lipid mixtures dispersed in chloroform and ethanol, (c): lipids dispersed in ethanol ready for liposome production

Figure 2.10 shows the steps we need to take after preparing the lipid mixture. It is important to carefully handle the waste, and all tools contaminated with chloroform and seal them thoroughly. The dispersed lipids in ethanol should be moved to vials and labeled properly providing information such as the lipid mixture, date of preparation and operator's name and stored in a -20°C refrigerator. The dispersed lipids in ethanol are good for use for up to two months.

2.2.2 Liposome production using milli mixer

Liposome production using millimeter mixers is an advanced technique for synthesizing lipid-based nanoparticles (liposome). Milli mixers, the scaled-up versions of micromixers, facilitate the mass production and efficient mixing of lipid and aqueous solvent to form liposomes. In the first step, to identify the samples, all Eppendorf tubes must be appropriately labeled. Afterwards, the heater from the hot plate (Purchased from Torry pines) is activated. In this

work, the required temperature for synthesis of liposomes is 40°C. In the next step, the prepared lipid mixture, which is described in detail in the previous subsection, should be removed from the refrigerator and placed on the heater alongside the milli mixer. To heat up the mixture and dissolve all the ingredients inside ethanol.

It is imperative to note that prior to introducing the lipids into the mixer, the lipids must undergo vortex mixing and be warmed to 40°C, as the melting temperature of DMPC is 26°C. Alternatively, the lipid mixture may be subjected to sonication (Cole-Parmer) for 15 minutes at 45°C (Zouggari, 2022). In Figure 2.11, sonicator used for mixing the lipid mixture in this study is displayed.

Figure 2.11 Sonicator for mixing the lipid mixture

To begin preparation of the structure, it is essential to completely clean the mixing channel of the structures to make sure there are no particles or clogging left from the previous runs. The

cleaning process involves two cycles; firstly, by using 10 mL of ethanol (99.9% Ethanol absolute CAS 64-17-5 purchased from Emdmillipore) as the organic solvent and 10 mL of MilliQ water as the aqueous solvent. Any air bubbles inside the syringes must be removed and a filter should be attached to the syringe before introducing the solvents to the pumps to prevent contamination.

As ethanol is an organic solvent, the syringe containing ethanol should be placed in the organic solvent pump. Conversely, MilliQ water is an aqueous solvent so it should be placed in the aqueous solvent pump. The settings of the pump during cleaning the milli structure include a force level of 50%, a pump speed of 120 mL per hour, and a total infusion volume of 178.58 ml. It is not essential to set a specific time because the cleaning process continues until the syringes are empty and the cleaning is completed.

On the other hand, we need to prepare material and the conditions for the liposome synthesis process while the mixers are cleaning. All the information concerning the volume and the amount of the ingredients, and the condition of synthesis are recorded in an Excel file, specifying the amounts of variables such as FRR, TFR, and PBS, collection time, the required volume of sample and aqueous solvent for each vial, and the temperature of synthesis. It is necessary to have a specific amount of aqueous solvent in each vial before collecting the liposomes in order to dilute them for further characterization process. It is essential to label carefully each vial (Eppendorf, 2000µl) to keep track of the collection time and setting the viables properly because the collection time, which basically means the time required to collect a specific amount of the synthesized liposomes, is different for each sample, and it is dependent to TFR. By increasing TFR, the total flow rate inside the channel increases, causing more fluid to pass it through the channel, which decreases collection time of the sample.

For the synthesis of liposomes, we filled a syringe with the lipid with a formulation of DMPC:CHOL:DHP, in a 5:4:1 ratio, diluted in ethanol as the organic solvent and introduced it to the organic pump. As the aqueous solvent, we used Phosphate-buffered saline (PBS)

which is an ionic solvent. In fact, we used PBS as the aqueous solvent to investigate its effect on liposome's physicochemical characteristics. A syringe was filled with PBS and introduced to the aqueous pump.

In the next step, we need to give all the data related to the synthesis such as FRR, TFR, collection time, Temperature and set a stabilization time of 90 seconds to the software that is running the pumps for each run. The software's main page is displayed in Figure 2.12. Each of the samples is repeated three times to make sure that the results are reliable.

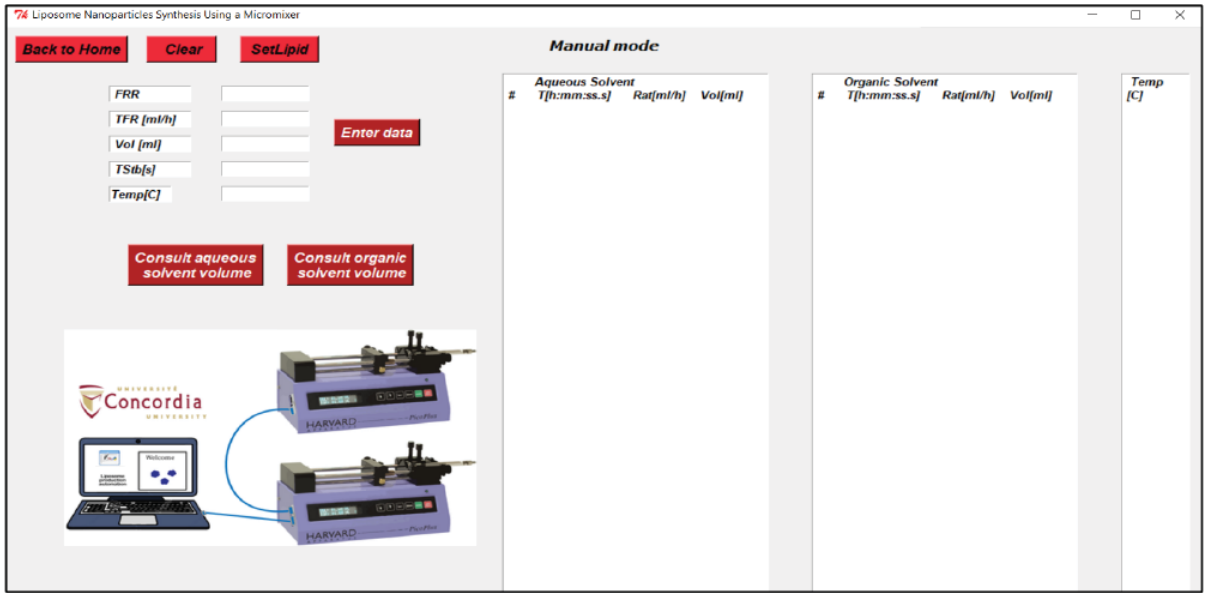


Figure 2.12 LipoSynthesis software

After running the system, before collecting each sample, we need to wait for 90 seconds for the flow and the conditions of synthesize to be stabilized (stabilization time), then the software will notify the collection time by an alarm. After collecting the first sample, the software stops pumps from pushing the syringes and we need to update the information with data related to the synthesis of the next sample.

2.3 Characterization of the liposome

After synthesis of liposomes, the physicochemical characteristics of liposomes, including Z-average (hydrodynamic diameter or intensity-based size mean in nm), Poly Dispersity Index (PDI or homogeneity of particle size), and zeta potential (mV), were assessed. The Z-average and PDI were measured using dynamic light scattering (DLS) principles and zeta potential and additionally mean size measurements were determined using the concept of electrophoretic mobility and it was measured using Zeta view device. The Zeta view device is a Nanoparticle Tracking Analysis instrument (NTA) which is also able to measure the size of the particle in very low concentrations. Dynamic Light Scattering (DLS) purchased from Malvern Panalytical and ZetaView PMX120 devices shown in Figure 2.13 have been used for characterization of the liposomes.

Figure 2.13 Devices for liposome characterization

In Figure 2.13 (a), there is a zeta view device and the related set-up and in (b) there is a DLS machine and its set-up. The principles of the devices are further discussed in the subsequent sections.

2.3.1 Z-average and poly dispersity index (PDI)

The Z-average is a crucial measure in the studies of nanoparticles, that exhibits a reliable and solid estimation of the average particle size suspended in a sample. It's particularly efficient for quality controls, meeting requirements, and comparing various formulations.
Z-average is a representative for a hydrodynamic diameter (Dh). Hydrodynamic diameter is defined as the diameter of a hypothetical hard sphere that demonstrates diffusion rates the same as the particle under analysis (Niskanen et al., 2019).

The definition of Z-average or intensity-based size mean is a measurement of the average size of the particles in a suspension. The Z-average is usually calculated using cumulant analysis of intensity autocorrelation functions of the scattered light intensity in Dynamic Light Scattering (DLS) measurements. DLS quantifies Brownian motion (random movements of the particles moving from regions with higher concentration to regions with lower concentration) and converts this data into the hydrodynamic diameter (Dh). It works based on an intensity-weight average, in which larger particles gain more weight as they scatter more light.

In nanoparticle characterization, the definition of Polydispersity Index (PDI) is the distribution of particle sizes in a sample. It can give us an insight into homogeneity or heterogeneity of the size of the particles in a sample. PDI quantifies the particle size distribution breadth, and the range is between 0 to 1. If the PDI is lower than 0.1 represents homogeneous or monodisperse particle size distribution. If PDI is between 0.1 to 0.3 the sample has moderately broad size distribution and if the PDI value is higher than 0.3 the sample has highly polydisperse size distribution with a wide range of particle sizes (Bhattacharjee, 2016). PDI is a fundamental parameter in nanoparticle characterization by which we can determine the homogeneity of sizes of the particle within a sample. PDI can impact the stability and performance of liposomes.

Dynamic Light Scattering (DLS) is a widely used method for measuring the Z-average and size distribution (PDI) of nanoparticles in a suspension. In order to prevent aggregation and disperse the particles well when preparing the samples, they should be diluted to a suitable concentration. Then we need to place the sample in a suitable cuvette. In Figure 2.14, two types of cuvettes used for Z-average and PDI are pictured.

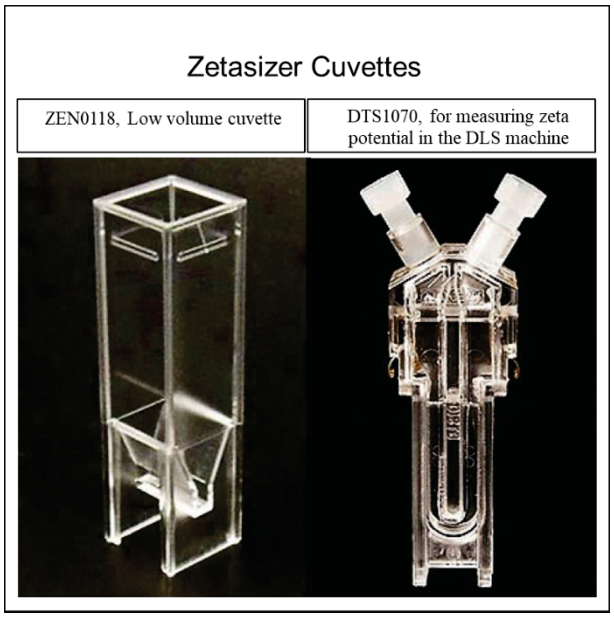


Figure 2.14 Cuvettes used in Zetasizer or DLS machine Taken from ZEN 0118 and DTS1070 (Panalytical)

In the following step, the cuvette should be cleaned completely as the laser beam is going through the cuvette and then inserted in the DLS machine. After starting the machine, the device emits a laser beam to pass through the sample and the particles inside the sample, which move randomly due to collisions with solvent molecules, will scatter the light. In this situation, smaller particles move faster than larger particles and because of this movement, the intensity of scattered light fluctuates over time. At a particular angle the scattered light is collected and subsequently a detector measures the intensity of the fluctuations. Using the analysis of the

correlation of light intensity over time, the autocorrelation function can relate these fluctuations to particle size.

Using autocorrelation data, diffusion coefficient (D) is derived. The diffusion coefficient has an inverse relationship to the particles size; smaller particles are moving faster and have a higher diffusion coefficient.

Using the Stokes-Einstein equation, the diffusion coefficient can be converted into the hydrodynamic diameter (Dh) of the particles which represents the size of the particles using formula 2.5 (Bhattacharjee, 2016):

$$D_t = \frac{k_b T}{6\pi\eta \mathrm{R_H}} \tag{2.5}$$

In above- mentioned formulation, D_t is translational diffusion coefficient, k_b represents the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and $\mathrm{R_H}$ represents the hydrodynamic radius, which is the effective radius of a particle in a fluid.

If we want to express the hydrodynamic diameter $\mathrm{D_h}$ of the particles instead of the radius, we require to use the relationship $\mathrm{D_h}=2\mathrm{R_H}$.

2.3.2 Zeta potential

Zeta potential is a key element in the characterization of nanoparticles. Zeta potential is basically defined as the electrical potential or the charge of the boundary layer that surrounds a nanoparticle (slipping plane of a particle) in a colloidal dispersion. The slipping plane specifies the outer boundary of the Stern layer, which as illustrated in Figure 2.15, consists of ions with opposite charges to the nanoparticle attached by electrostatic interactions to it. PH, the presence of surfactants or polymers, and ionic strength are some of the factors that can influence zeta potential. Zeta potential is a crucial parameter in designing stable formulations for drug delivery. As Zouggari et al. indicated in her project, by increasing

the magnitude of the zeta potential (positive or negative), the stability of the suspension is increased, as the forces of repulsion between the colloids increase, preventing particle aggregation (Herrada García, Pérez Corona, Shrestha, Pamukcu, & Bustos, 2014; Zouggari, 2022).

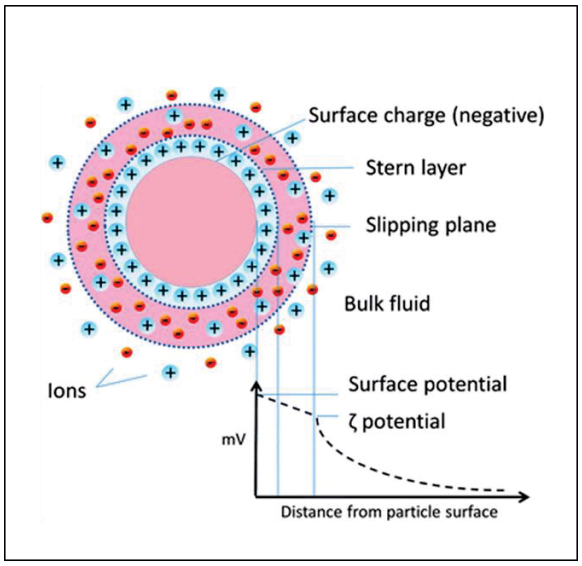


Figure 2.15 The electric double layer configuration
Taken from (Herrada García et al., 2014)

Zeta potential measurements are achieved by using particle electrophoresis defined as Electrophoretic Light Scattering (ELS). The principle of ELS technique is based on particle movement which is achievable by applying an electric field to the colloidal suspension. An oscillating electric field triggers the particles' Brownian motion and the electrophoretic movement of the particles, μ (m²/Vs), could be calculated as follows:

$$\mu = \frac{v}{E} \tag{2.6}$$

Where E is the electric field strength and v is the electrophoretic velocity of the particles that can be converted to zeta potential(Charcosset, 2016). The Zeta Potential (ζ, mV) finally is measured using the following equation which is named as Smoluchowski equation:

$$\zeta = \frac{\mu 4\pi\eta}{\varepsilon} \tag{2.7}$$

In this equation η its viscosity of the medium and ε is the medium permittivity (Zouggari, 2022). This equation also shows that zeta potential has a direct relationship with η and μ, but it has a reverse relationship with ε. It is necessary to indicate that the permittivity (ε) of PBS (phosphate-buffered saline) is typically around $78\epsilon_0$, slightly lower than the permittivity of pure water which is $80\epsilon_0$. This lower value of the permittivity is because of the presence of ions in the PBS which would conclude in higher amounts of zeta potential. The zeta potential basically indicates the stability of colloidal dispersion and is highly influenced by the medium's permittivity.

All of the measurements of zeta potential in this work were conducted at 25°C unless stated otherwise, and three different pieces of equipment were used: ZetaPlus (Brookhaven Instrument Corp.) and Zetasizer Nano Pro (Malvern Panalytical), which was used to measure Z-average, PDI and Zeta Potential at UQAM, and ZetaView (Particle Metrix) which was used to measure zeta potential basically and Z-average as well at MUHC. All of the measurements were replicated at least 3 times to ensure the accuracy and reliability of the results. In Figure 2.16, a capillary cell is displayed which contains two gold-plated electrodes. These electrodes can create the electric field to make microelectrophoresis happen as they are in contact with the diluted particles inside the bulk material.

Figure 2.16 Capillary cells used in Zetasizer Nano pro for microelectrophoresis Taken from (Panalytical)

The ZetaView instrument uses NTA to measure characteristics of the nanoparticles such as the size, zeta potential, and concentration which is displayed in Figure 2.17. It combines electrophoresis, video capture, and laser light scattering to track the Brownian motion of each particle and investigate the characteristics. This device makes charged nanoparticles move by applying an electric field to the sample. The instrument measures electrophoretic mobility by observation of the speed and direction of the nanoparticle movement in response to the electric field. Using the Smoluchowski equation, the electrophoretic mobility of the particles converts into the amount of the zeta potential.

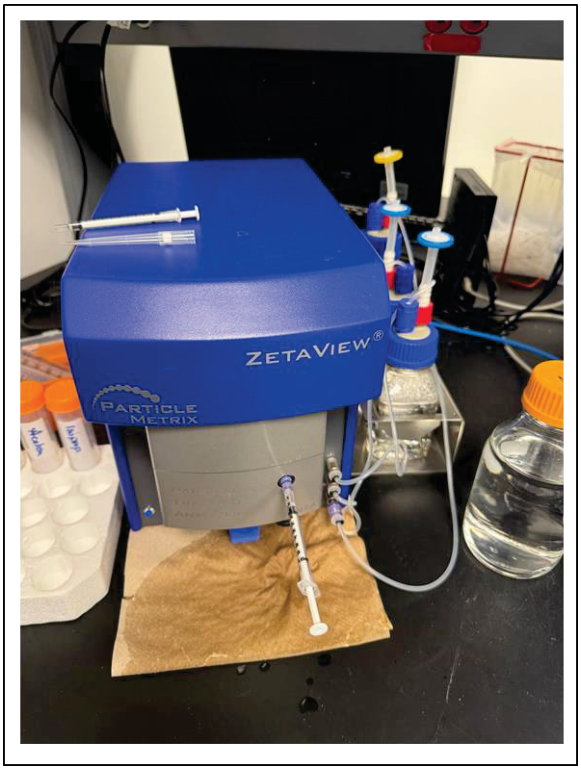


Figure 2.17 ZetaView device

For sample preparation, obtaining an appropriate concentration by diluting the nanoparticles is crucial. The sample should be free of air bubbles and the instrument's sample chamber should be cleaned by injecting MilliQ water. In the following step, the sample should be injected gradually. As the laser is illuminated, nanoparticles start to scatter light and motion of the nanoparticles could be captured using a camera. The software is able to calculate particle size distribution from the Brownian motion data. Figure 2.18 is displaying tools required for ZetaView analysis.

Figure 2.18 Tools required for ZetaView analysis; including sampler 100 and 1000 µl purchased from Eppendorf and Gilson, MilliQ water for dissolving the samples, Eppendorf's, BD 1 ml syringe

Aiming to measure zeta potential, measuring the electrophoretic mobility is required, that has been done by applying an electric field to move the charged particles. Then the speed and direction of this movement is measured by the instrument.

2.4 Chapter 2 conclusions

In this chapter, all the materials and methodologies used to design and print the milli mixers with high mixing efficiency suitable for producing liposomes with physicochemical characteristics such as surface charge (Zeta Potential) and hydrodynamic diameter (Z-average) and PDI suitable for industrial scale, were presented. In Figure 2.19, a summary of the steps involved in this methodology is depicted in detail.

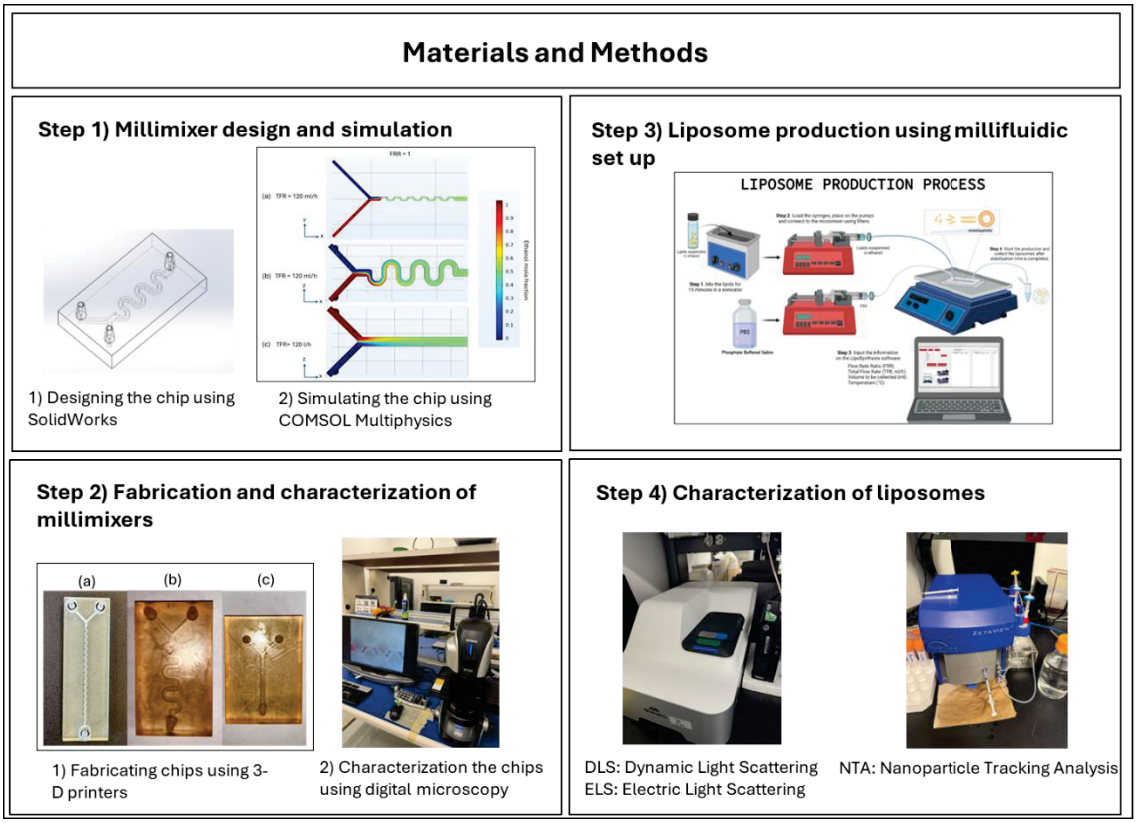


Figure 2.19 A schematic of materials and methods

CHAPTER 3

DESIGN AND FABRICATION OF MILLI MIXERS: RESULTS AND DISCUSSION

This chapter will illustrate simulations and strategies carried out to make milli mixers that allow synthesize liposomes in higher volumes and increase the production yield while trying to maintain liposomes' size and zeta potential.

The milli mixers were designed to facilitate synthesis of lipid-based nanoparticles with an efficient mixing at a larger scale in comparison with the micromixers. The channel dimensions range in millimeter mixers is different from hundreds of micrometers to millimeters. The design incorporated Y-shaped inlet channels connected to a straight or semicircular structures designed in alternating directions to fabricate a mixing channel where the synthesis of nanoparticles happens. The goal of these configuration and designs is to enhance mixing efficiency by taking advantage of inducing secondary flows and improving fluid interaction. The key in the design optimization process is to consider the need for scalability while maintaining effective mixing.

The design of the mixer should ensure to handle larger fluid volumes without compromising performance. Simulations of the designs before fabricating the structure and after that are crucial to estimate and verify mixing efficiency within the mixing channel. Simulation in COMSOL Multiphysics® is fulfilled to obtain mixing efficiency inside the channel before printing the structure and flow visualization techniques, such as dye tracing using digital microscope, were employed to assess the mixing performance inside the channels after printing the structure. The analysis of flow patterns and visualizing mixing phenomenon is obtained by the data gathered from these techniques in formats such as estimating mixing efficiency, high-resolution imaging and video recording. These steps including design, simulations and experiments would be described thoroughly in the following sections.

3.1 Design and simulation of three different milli mixers

The mixing phenomenon in fluidic mixers is the dispersion of dissolvent species to obtain uniform solution. One of the examples is in nanoprecipitation. Nanoprecipitation is the phenomenon that transforms lipids into liposomes, and it is based on the mixing of a low-polarity organic solvent with a high-polarity aqueous solvent. Due to Taylor Dispersion, fluidic devices operate with laminar flow. Taylor Dispersion happens when viscous forces dominate inertial forces and the fluid flows smoothly, meaning that there is a velocity gradient in the flow that results in the diluted species. Therefore, under constant boundary conditions, a linear velocity of the fluid flow at all cross sections of the channel occurs. In the following steps, through chaotic advection, molecular diffusion and Taylor Dispersion mixing predominately occurs.

When the flow path is curved, or many obstacles are embedded in the mixing channel chaotic flow occurs. Chaotic advection influences the mixing by inducing early moving fluid movement and chaotic flow, but it should be under laminar flow conditions. Molecular diffusion occurs by the molecules and particles movements in a fluid with different concentrations, from high to low concentrations. Different conditions can affect this movement such as temperature, the size of the particles and liquid viscosity and it is defined by Fick's first law. On the other hand, turbulent flow happens when mass transport in all directions and varies in space and time and random motion of the molecules happens. In this occasion, internal forces are dominant to the viscous forces. The Reynolds number (Re) can help us to determine laminar flow vs turbulent flow, and it is calculated by the following formulation:

$$Re = \frac{\rho u D_h}{\mu} = \frac{u D_h}{v} \tag{3.1}$$

In this formula, u represents for the flow velocity, ρ is a representative of the fluid density, v is kinematic viscosity, μ represents dynamic viscosity, and D_h is a hydraulic diameter. In Table 3.1, different ranges of Reynolds number and their characteristics are defined separately:

Table 3.1 Reynolds number range and related flow regimes

Flow Regime	Reynolds Number (Re) Range	Characteristics
Laminar Flow	Re < 2000	Fluid moving smoothly, Orderly layers, Predictable and stable flow.
Transition Flow	Re ~ 2000 - 4000	Flow begins to transition from laminar to turbulent, Increased mixing, avoiding the complexities of fully turbulent flow.
Turbulent Flow	Re > 4000	Significantly enhances mixing efficiency, Introduction of challenges in controlling the flow and ensuring uniform mixing.

To guarantee laminar flow regime, in which fluid moves inside the channel smoothly, we need Reynolds number to be as small as possible that results in stable fluid flow interfaces which is a factor for preparing homogenous liposome populations. Although turbulent flow interface can provide higher mixing than purely laminar flow, it makes it challenging to control the flow and ensure uniform mixing. Typically, to ensure laminar flow, Reynolds number should be under 2000. Besides that, to increase production yield, the cross-section dimensions have been increased which needs further optimization of flow while maintaining the same temperatures for the synthesis process.

In addition to Reynolds' number, it is important that the speed of the fluid passing inside the channel be as fast as possible to induce dean vortices. Dean vortices can enhance mixing efficiency which is one of the most important factors in fluidic systems. Dean number must be 10 or higher to achieve high mixing efficiency in fluidic system. Therefore, there are two requirements in fluidic system to create effective transverse mixing and maximize the efficiency and yield of the synthesis process; Reynolds number which should be kept as low as possible to ensure laminar flow and the design of the mixing channel to provide the necessary flow speeds.

3.1.1 Scaling up the mixers from micro to milli

In order to design and evaluate the mixing efficiencies and fluidic properties in the mixing channel of milli mixers, three separate designs were chosen and simulated using simulation software. We chose three different existing micro mixers and tried to scale up their mixing channel dimensions to design and further fabricate the millimeter-scale mixers.

The designs of a Y-shaped and Serpentine micrometer mixers were derived from the work of Tripathi et al. and Karthikeyan et al., following by scaling them up using SolidWorks by factors of 3, 5, and 10 (Karthikeyan & Sujatha, 2019; Tripathi, Patowari, & Pati, 2023). The Y-shaped design consists of two inlets coming together to make a V shape design connecting a straight mixing channel. The serpentine design consists of a V shape of two inlets connecting to a mixing channel with curved shaped structure. This design helps us to yield efficient processing and thus increases the chances for mixing.

As the third milli mixer, we chose the design of the Periodic Disturbance micromixer (PDM) from Ruben et al. In this case, the mixing channel consists of a V shape of two inlets connecting to a mixing channel with 40 semicircular structures, which are arranged in alternating directions, with a radius of 260 μm and a maximum width of 300 (López, 2020). This mixer consists of a multi-stream with obstacles (semi-circular structures) to enhance the mixing and impose chaos mixing within the devices. In Figure 3.1, the designs and the dimensions of these three micromixers are depicted.

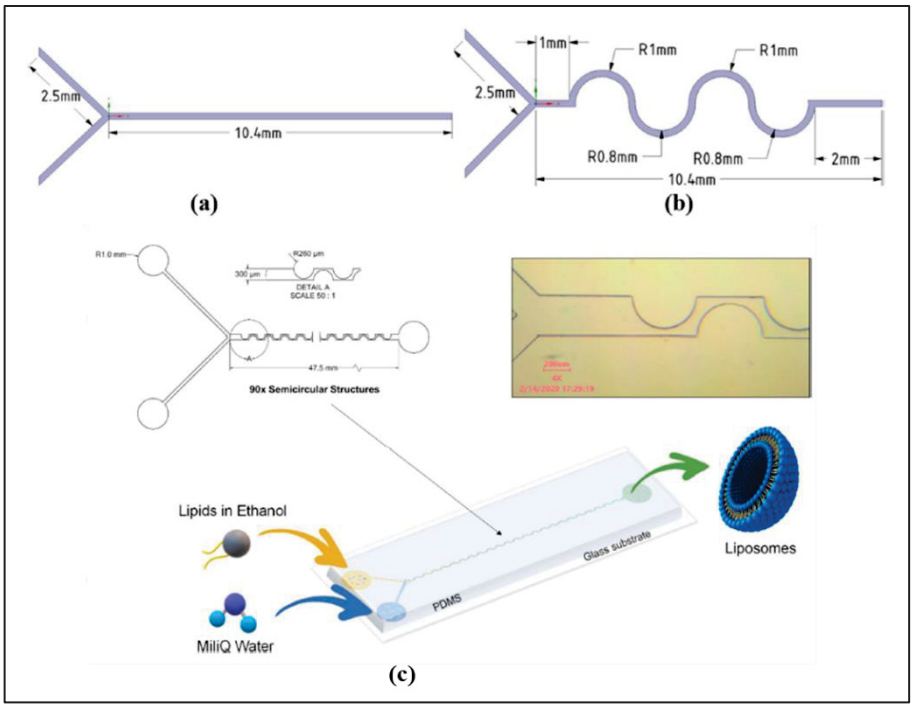


Figure 3.1 Original designs and dimensions of the micromixers used in this work Taken from(R. R. Lopez et al., 2020; Tripathi, Patowari, & Pati, 2023)

All the cross-sectional dimensions of the three designs in this work were scaled up by a factor of 10 in SolidWorks. In Figure 3.2 schematics of the three designs are provided.

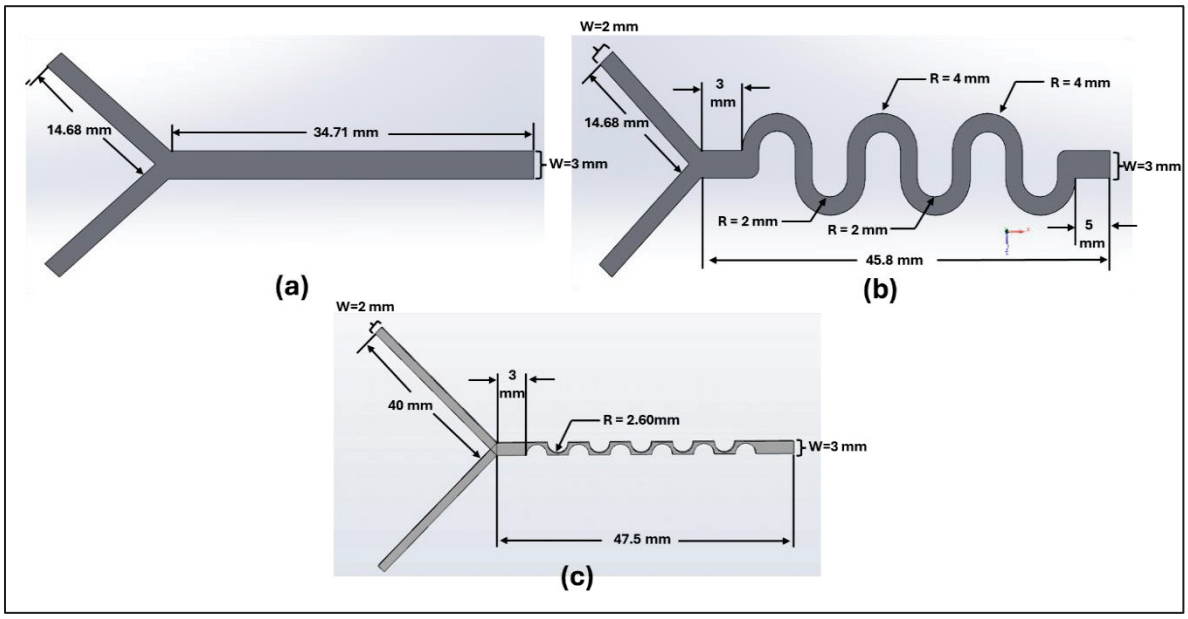


Figure 3.2 Schematic of (a) straight, (b) serpentine, and (c) PDM milli mixer designs after scaling them up by a factor of 10

As illustrated in Figure 3.2, the mixing channels of these three milli mixers are scaled up to 10 times larger compared to their original micro mixer size, as displayed in Figure 3.1. The width of the mixing channel is 3mm in all these milli mixers, however the width of the semicircular structures in Serpentine and PDM decreased to 2mm for Serpentine milli mixer and 0.4 mm for PDM milli mixer.

In order to scale up the design of milli mixers from the existing micromixers, we used different scale-up factors. In fact, the cross-sectional dimensions of three designs were scaled up by factors of 3, 5, 10, and 100. The purpose of scaling up with four different factors was to evaluate the mixing efficiency for each of the factors to find the most optimal factor for the synthesis process. Finally, the choice of a factor of 10 was made based on the results obtained from these evaluations. It is essential to estimate the mixing efficiency inside the mixing channel to understand which dimension is more optimal for synthesis process before using the milli mixer in the laboratory. Numerical simulations are also useful in determining at which point of the mixing channel the efficient mixing occurs. This information is beneficial for designing and optimizing dimensions of milli mixer to enhance liposomes' mass production

while maintaining their desired physicochemical properties and mixing efficiency inside the mixing channel. To achieve this purpose, all milli mixer designs were uploaded to COMSOL Multiphysics® 6.1 for simulating the liquid flow and estimating the mixing efficiency of the channels and visualizing mixing phenomenon. Figure 3.3 displays simulations of the PDM and Serpentine and Y-shaped milli mixers.

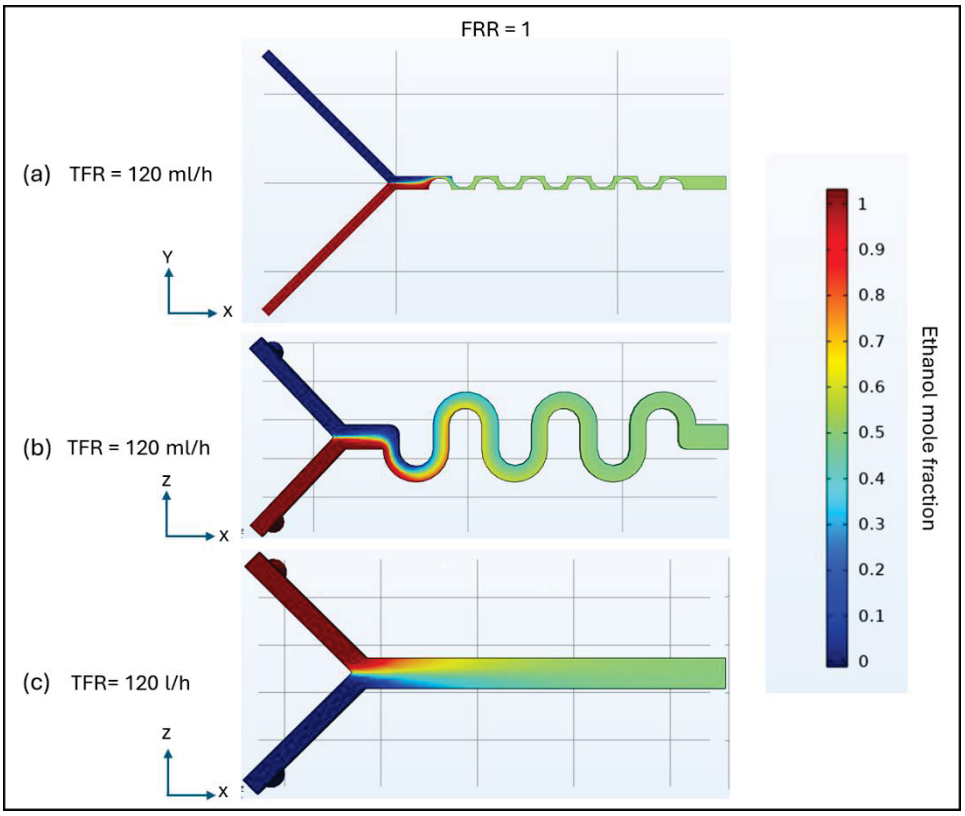


Fig 3.3 Simulation of Y-shaped, Serpentine and PDM milli mixers

Figure 3.3 displayed each milli mixer is designed and scaled up in a way to yield high mixing efficiency for synthesis of the liposomes and the green color at the outlet of these simulations indicates that the mixing phenomenon is happening within the mixing channel. The mixing efficiency of the mixing channel using numerical simulations in COMSOL Multiphysics® is calculated and defined in detail in the next section.

3.2 Flow visualization and mixing efficiency in milli mixers

Optimizing the process of synthesis of liposomes using millimeter mixers requires understanding thoroughly flow dynamics and mixing efficiency phenomenon. Milli mixers with larger scales of mixing channels are able to maintain high mixing efficiencies while at the same time offer advantages in scalability and mass production. In this section we will focus deeply on estimating mixing efficiency using COMSOL Multiphysics® and visualizing mixing using digital microscopy inside the PDM milli mixer channels as we aim to compare the results of the evaluation obtained from PDM milli mixer to the performance of traditional PDM micromixer studied by Lopez et al (López, 2020). By investigating mixing indices and flow patterns happening in the mixing channel under various FRR and TFR conditions, this study reinforces the benefits of milli mixer designs in the process of liposome production.

3.2.1 Calculating mixing efficiency using COMSOL Multiphysics®

Mixing efficiency is one of the most important parameters specifies whether a fluidic mixer is suitable for the process of synthesis of liposomes or not. In this section, mixing is modeled numerically using COMSOL Multiphysics® 6.1 by solving a coupled equation of convection-diffusion equation with Navier-Stokes equations, considering a single-phase flow. These equations were repeatedly solved in the simulation until the system reached a steady state.

$$\rho(u.\nabla)u = \nabla.[-pI + \mu(\nabla u + (\nabla u)^T)] + F \tag{3.2}$$

$$\rho\nabla.(u) = 0 \tag{3.3}$$

In this equation ρ represents the fluid density, p is pressure, u is a representative of the flow velocity, μ is dynamic viscosity and F is outer forces. The walls of the model were set to no-slip boundary conditions. The amount of the velocity field obtained from the above-mentioned equation was utilized to resolve the convection-diffusion equation:

$$\nabla.(-D\nabla c) + u.\nabla c = R \tag{3.4}$$

$$N = -D\nabla c + uc \tag{3.5}$$

In this equation c demonstrates the concentration of the diluted species, D represents the mutual diffusion coefficient between ethanol and water, N is molar flux, and R represents the net volumetric source for the species. D, ρ, and μ in the previous equations depend on the solvent concentration c in the aqueous medium. This approach is proficient in modeling a binary mixture using a single-phase liquid with changing fluid properties (R. R. Lopez et al., 2020).

To calculate Mixing Efficiency (ME) inside the channel, cross sectional planes were sketched perpendicular to the main flow direction and each plane was divided into 100x100 grid. In the following step, the concept of Danckwerts intensity of segregation was applied to the concentration data derived from these grid elements. Then using following equation, ME calculated:

$$ME = \left[1 - \sqrt{\frac{\sigma^2}{{\sigma_0}^2}}\right] \times 100\% \tag{3.6}$$

Where ${\sigma_0}^2$ is a representative for the concentration variance at the beginning of the mixing channel, where liquids joining together and they are not mixed yet, and σ^2 is the concentration variance at a desired cross-section. In Figure 3.4, the cross-sectional planes are defined at various places along the mixing channel from the starting point.

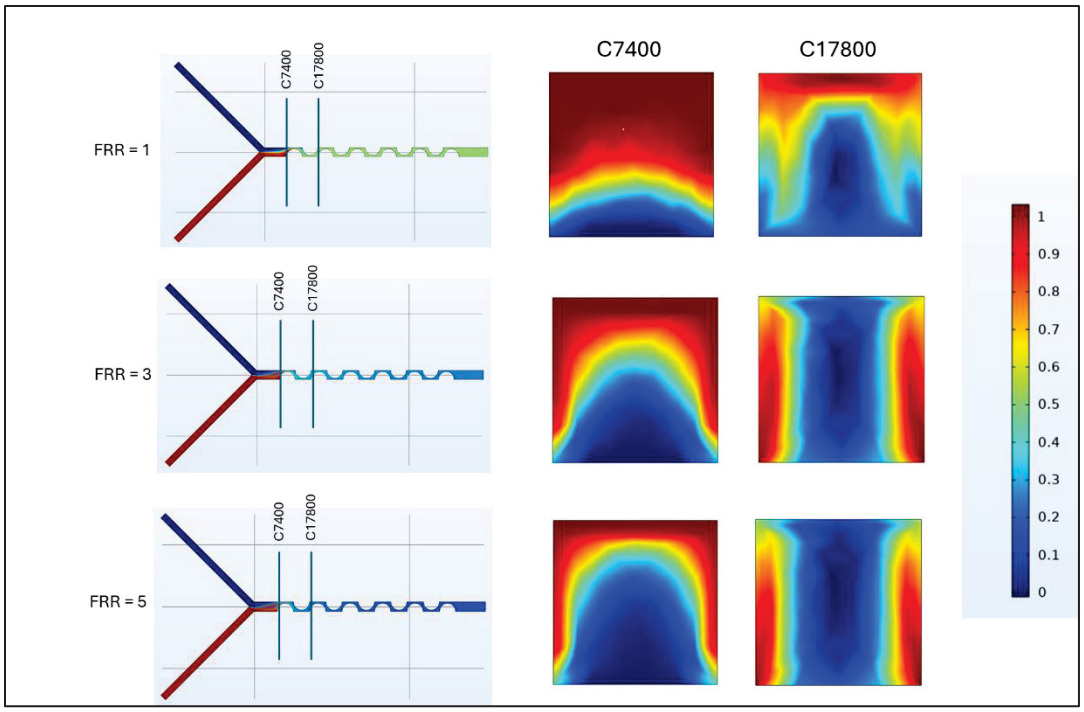


Figure 3.4 Numerical simulations derived from COMSOL Multiphysics comparing concentration profiles at FRR=1, 3, 5 and a constant TFR=120 mL/h

Our main objective from simulation and calculating mixing efficiency in this section is to compare the micro and milli mixers of PDM and therefore in Figure 3.4, two cross-sectional plans are shown perpendicular to the mixing channel of PDM milli mixer for each FRR at a constant TFR value of 120 mL/h. On the left we can see the position of the cross-sectional planes on the mixing channel of PDM milli mixer from the upper view. Each of them is positioned in different places of the mixing channel to facilitate measuring mixing efficiency. The first plane named “C7400” is placed with 7400 µm from the beginning of the mixing channel and the second plane is positioned after two loops with 17800 µm from the starting point of the mixing channel, which is defined as “C17800”.

The cross-sectional pictures from left to right show by moving further inside the mixing channel, the mixing is increasing.

Each of these cross-sectional plans was consider as a greed of 100x100 and all the data related to the concentration was gathered from those planes and based on mixing efficiency formula which was mentioned above (3.6), the mixing efficiency was measured and put in a chart. Figure 3.5 is the chart resulting from the mixing efficiencies at different distances.

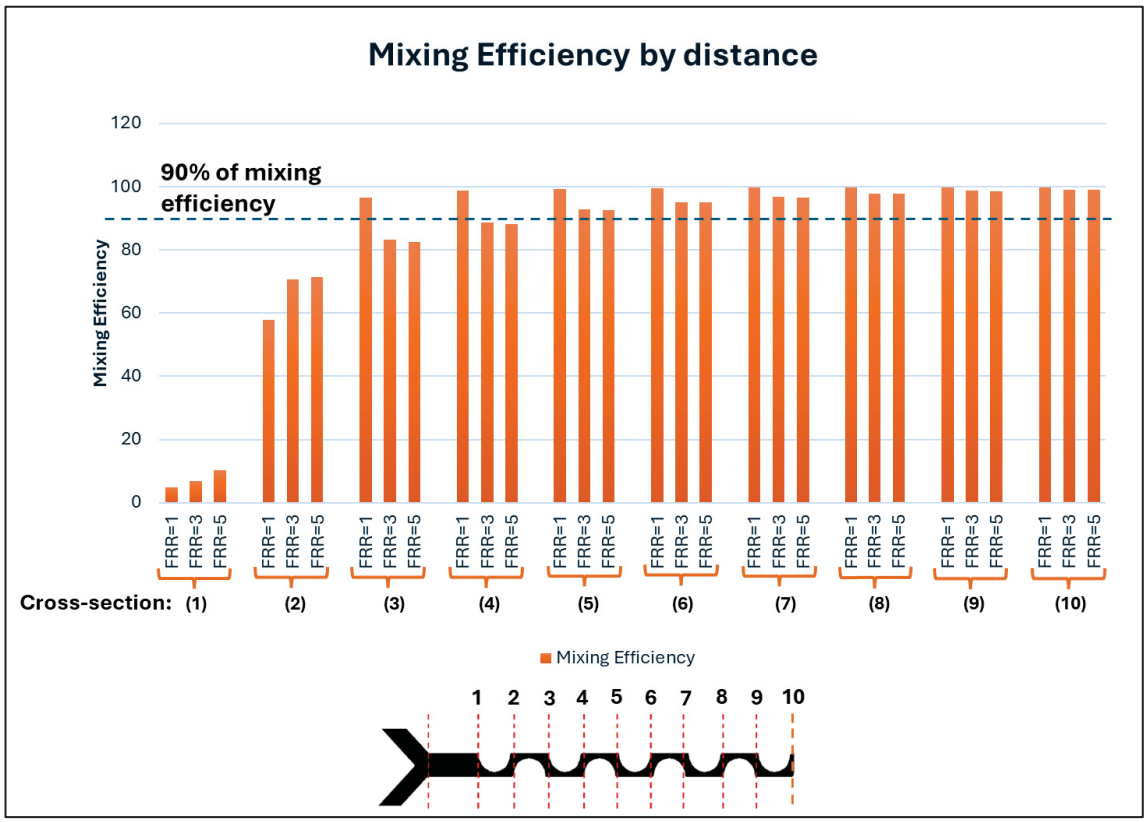


Figure 3.5 Mixing efficiency at different FRRs

In Figure 3.5, the chart of the mixing efficiency by distance is displayed in which each of the three data bars display the amount of Mixing Efficiency (ME) in three different FRRs corresponds to a cross-section. As we can see by increasing the distance from the starting point of the mixing channel, mixing efficiency is increasing. In cross-section number 5 the mixing efficiency is higher than 90%, which indicates that the mixing process is highly effective. This level of mixing efficiency results in almost uniform mixing substances within all the mixture.

At this level of mixing, we can achieve almost complete blending of the solvents inside the system.

Mixing efficiency is known as a key factor for liposomes' stability and effectiveness in pharmaceutical and drug delivery applications as it can influence liposomes' size and distribution drastically (Andra, Pammi, Bhatraju, & Ruddaraju, 2022).

3.2.1.1 Comparing the results of mixing efficiency in PDM micromixer vs milli mixer

In this subsection, the results of the mixing efficiency of PDM milli mixer would be compared to the results from Lopez's PDM micromixers. In Figure 3.6, the results of mixing efficiency at different FRRs of Ruben's PDM micromixer are indicated.

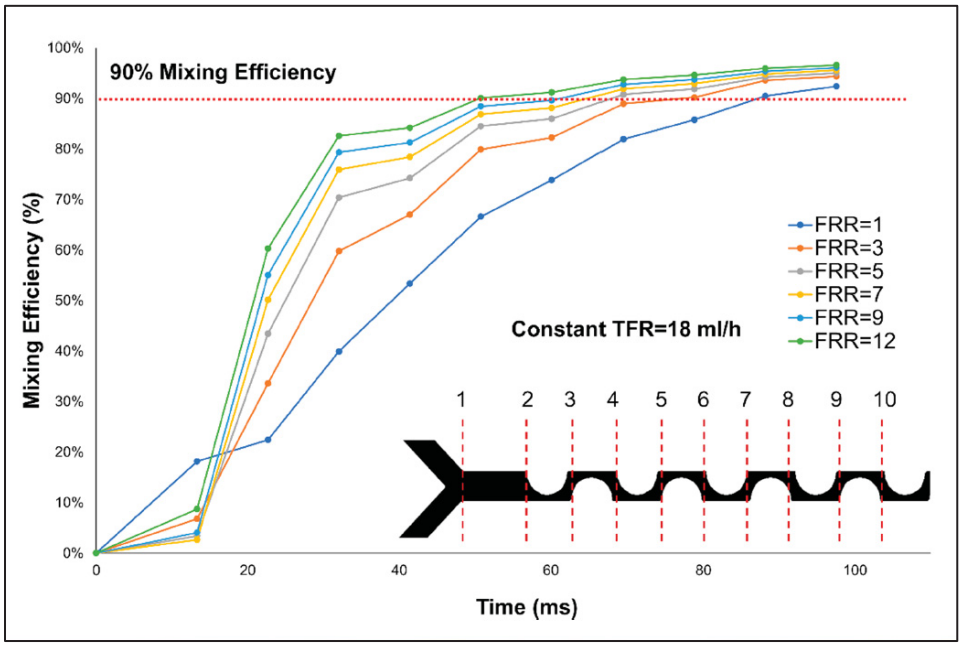


Figure 3.6 Mixing efficiency at different FRRs in PDM micromixer, taken from (R. R. Lopez et al., 2020)

Respectively, in Figure 3.7, the results of mixing efficiency at different FRRs from PDM micromixer are provided.

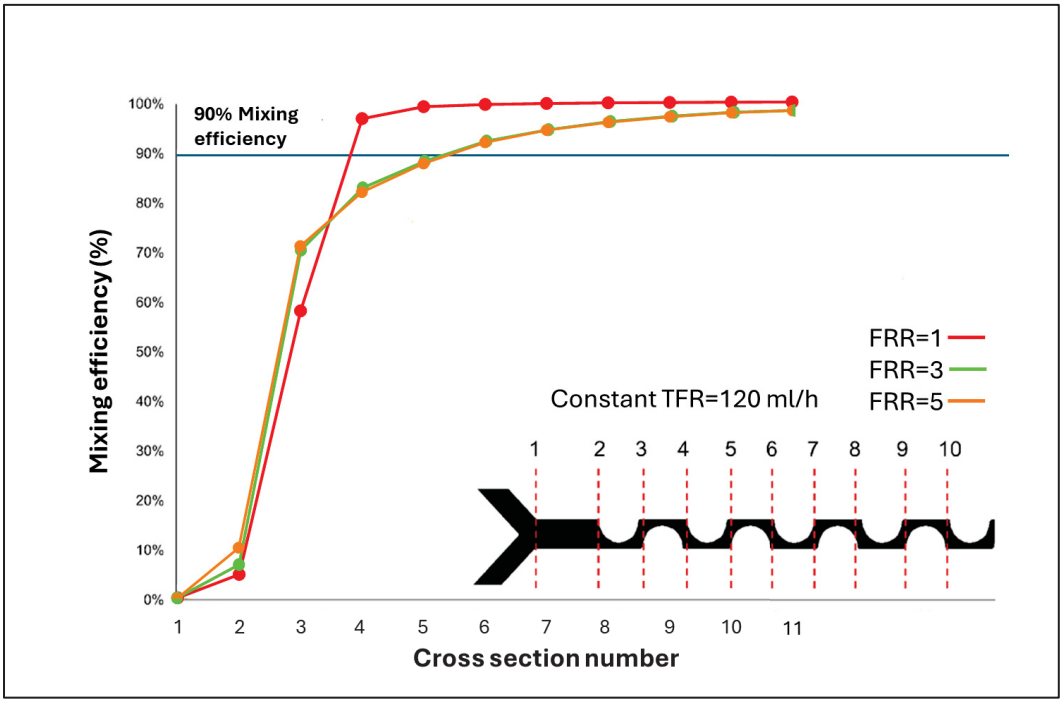


Figure 3.7 Mixing efficiency at different FRRs in PDM milli mixer

As Figure 3.6 displays, at FRR=5 and constant TFR=18 ml/h, the mixing efficiency reached above 90% after 6 loops (6 semicircular structure), and at FRR=1 and TFR=18 ml/h this time increased to 8 loops. On the other hand, as demonstrated in Figure 3.7, in PDM milli mixer at FRR=1 and constant TFR=120 ml/h mixing efficiency reached above 90% after two loops and with the same TFR in FRR=5, this time increased to only 4 loops.

These results indicate that at equivalent FRR and TFR values, mixing efficiency is superior in PDM milli mixer in comparison with the micromixer. This is because of the larger cross-sectional area of the milli mixers and their enhanced flow dynamics. The enhanced shear forces and turbulence inside the mixing channel can promote mixing efficiency. By maintaining

higher mixing efficiencies, scaling up for mass production of milli mixer without compromising the quality of the final products.

3.2.2 Flow visualization using digital microscopy

Observations of flow patterns within milli mixers are performed using digital microscopy. Digital microscopy makes it possible to directly observe the fluid flows, displaying flow uniformity, laminar and turbulent regimes, and phase interface. This information will help to optimize operational parameters to increase mixing efficiency and synthesis of uniform liposome.

Figure 3.8 Digital microscope (KEYENCE, VHX-7100) from ETS university

In Figure 3.8, we can see the set-up of the digital microscope from ETS, which is an optical microscope. To perform efficiency measurements for the experimental part of the test, two different dyes (red and blue) were used to inject inside the mixing channel for taking photo and videos when the mixing was happening. Before connecting the dyes to the milli mixer, we used 0.2-micron filters to prevent clogging and undesired fluctuations inside the mixing channel. In this experiment, 11 elite pumps were used which is the same pump used for synthesis of liposomes for the results to be reliable and comparable. This pump allows a highly precise flow rate adjusting and controlling.

After loading different food grade dyes and running the pumps, the videos were taken along the mixing channel revealing distinct flow patterns at different Total Flow Rates (TFR) and Flow Rate Ratios (FRR). The flow was predominantly laminar at lower FRR values, but it began to show more complex and turbulent patterns by increasing FRR values. This can optimize the mixing efficiency of the liquid inside the channel and increase the interfacial contact between the lipid and aqueous phases to promote synthesis of more uniform liposome populations. On the other hand, higher TFR values displayed more uniform flow patterns alongside the mixing channel. This observation suggests higher mixing efficiency and better distributions of shear forces happens at higher TFRs. Figure 3.9 displays the mixing happening inside the mixing channel.

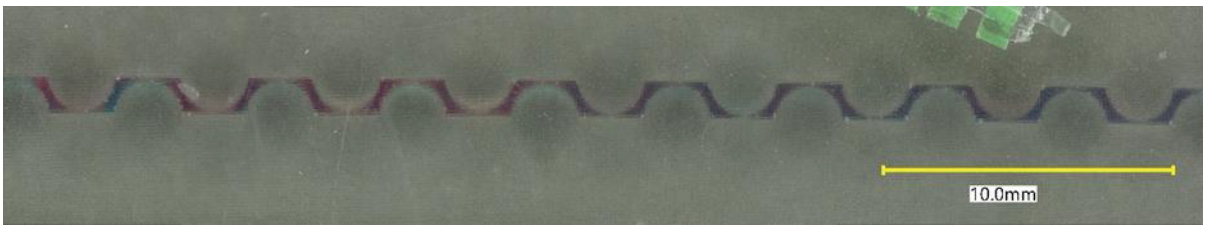


Figure 3.9 The picture of mixing blue and red dyes inside the PDM milli mixer with FRR=5 and TFR=120

In Figure 3.9, from left where inlets are placed to right where the outlet took place, the mixing is happening inside the channel. As we used red and blue colors to observe the mixing phenomenon, the mixture started to get a purple color after passing almost 9 semicircular structures of the mixing channel indicating that complete mixing has occurred.

We can compare this result from digital microscope experiment to the results of simulation of PDM milli mixer using COMSOL Multiphysics®. As Figure 3.7 displays, the results of mixing efficiency at FRR=5 reached 90 % of mixing efficiency after 3 semicircular structures and this amount get close to 100 % after 9 semicircular structures indicating that the complete mixing is happening. This is the point of the channel where at constant TFR = 120 ml/h and FRR = 5, we got the same results from both experimental and simulation experiments proving that the results are reliable.

3.3 Chapter 3 conclusions

In this chapter the investigation of the milli mixer design and simulation has been done. The investigations indicated that the design should maintain low Reynold number which is required for laminar flow within the mixing channel. Additionally, high values of shear forces are required to enhance mixing efficiency which is resulting in homogeneous liposome production which could be achieved by sufficient flow speed.

To investigate the flow patterns and mixing dynamics within the mixing channels of milli mixers, the mixing efficiency of PDM milli mixer was compared to the mixing efficiency of PDM micromixer. A digital microscopy and Numerical modeling using COMSOL Multiphysics® were fulfilled. Both results of the simulation and experimentation (microscope) were the same proving the reliability of the results. Mixing efficiency

enhancement was observed by increasing TFR and FRR values which highlights the importance of optimization of flow parameters for production of liposomes in an effective way. Milli mixers demonstrate reliable mixing performance which makes them a promising choice for efficient and scalable production of liposomes.

CHAPTER 4

PARAMETRIC INVESTIGATIONS OF THE VARIOUS FACTORS AFFECTING LIPOSOME PHYSICOCHEMICAL PROPERTIES: RESULTS AND DISCUSSION

As described in previous sections, liposomes have a spherical shape consisting of phospholipid bilayers which are widely used in biomedical and pharmaceutical applications. Because of the existence of phospholipids in their structure which have both hydrophobic tails and hydrophilic heads, they can encapsulate both hydrophilic and hydrophobic drugs.
The liposomes' physicochemical characteristics include zeta potential, polydispersity index (PDI), and size determining therapeutic efficacy of the liposomes such as their stability, biocompatibility and encapsulation efficiency.

Drug encapsulation efficiency of liposomes depends on liposomes zeta potential which affects liposomes' stability and their interactions with biological systems. For consistent drug loading and controlling drug release, it is important to have homogeneous liposomes. A lower PDI can result in more uniform size distribution liposomes. Size optimization of liposomes can influence drug encapsulation efficiency and facilitate drug penetration in tissue by increasing the surface area to volume ratio. To obtain optimized liposomes with desired characteristics, it is important to understand the factors affecting these characteristics. Different parameters such as concentration of PBS, flow rate ratios (FRR), total flow rate (TFR), and the design of mixing channel have huge impacts on the properties of the final product.

This section delves into investigating these different factors influencing physiochemical characteristics of liposomes. All the results obtained from three types of milli mixers (Y-shaped, serpentine and PDM) in different experimental setups are investigated to find a common trend in the effect of different factors influencing the final product. We aim to bring an insight into the influence of channel design and different factors on size, PDI and zeta potential of liposomes to optimize mass production of liposomes for pharmaceutical and industrial applications.

4.1 Effect of different FRRs and TFRs on liposomes' Z-average

Evaluating the effect of Flow Rate Ratios (FRRs) and Total Flow Rates (TFRs) on physiochemical characteristics of liposome is essential to optimize the synthesis conditions. As previously described, FRR indicates the ratio between the speeds of introducing the aqueous solvent and organic solvent to the fluidic devices, which influences how well these two solvents mix. This factor, in turn, affects the liposomes' Z-average. TFR affects the flow dynamics and how the fluids move within the mixing channel which influences the interaction of the fluids and the amount of time these interactions last during liposome formation. By controlling FRR and TFR, it is possible not only to investigate their effect on size of liposomes but also to scale up synthesis of liposomes using milli mixers.

Aiming to investigate the effect of these factors in this study, three different amounts for FRRs; 1, 3, 5 and respectively TFRs; 30, 60, 120 were choose and the liposomes were synthesized under 25℃ and each sample was produced three times with the same conditions for the results to be reliable. Therefore, 27 samples per milli mixer were produced and characterized using DLS and NTA approaches. Most of these replicates display results within the same range, showing consistency in the process of liposome synthesis. However, in some cases there are some variations indicating the experimental errors for minor fluctuations in the synthesis conditions.

The charts of Z-average, PDI and zeta potential of all 81 samples produced with three milli mixers are provided in the appendix (Figure-A I-1 and Figure-A I-2). To facilitate the comparison and investigation between the results of 27 samples for each type of milli mixer, we considered a mean value for results of Z-average, zeta potential and PDI of each three replicates with common synthesis variables and a variance to display the upper and lower values. This way, we have 9 samples with different TFRs and FRRs for each milli mixer, which facilitates investigating the effect of variables on the physicochemical characteristics of liposomes. Figure 4.1 displays a chart comparing Z-average of liposomes synthesized in three

different milli mixers. The measurements were conducted using Dynamic Light Scattering (DLS) instrument.

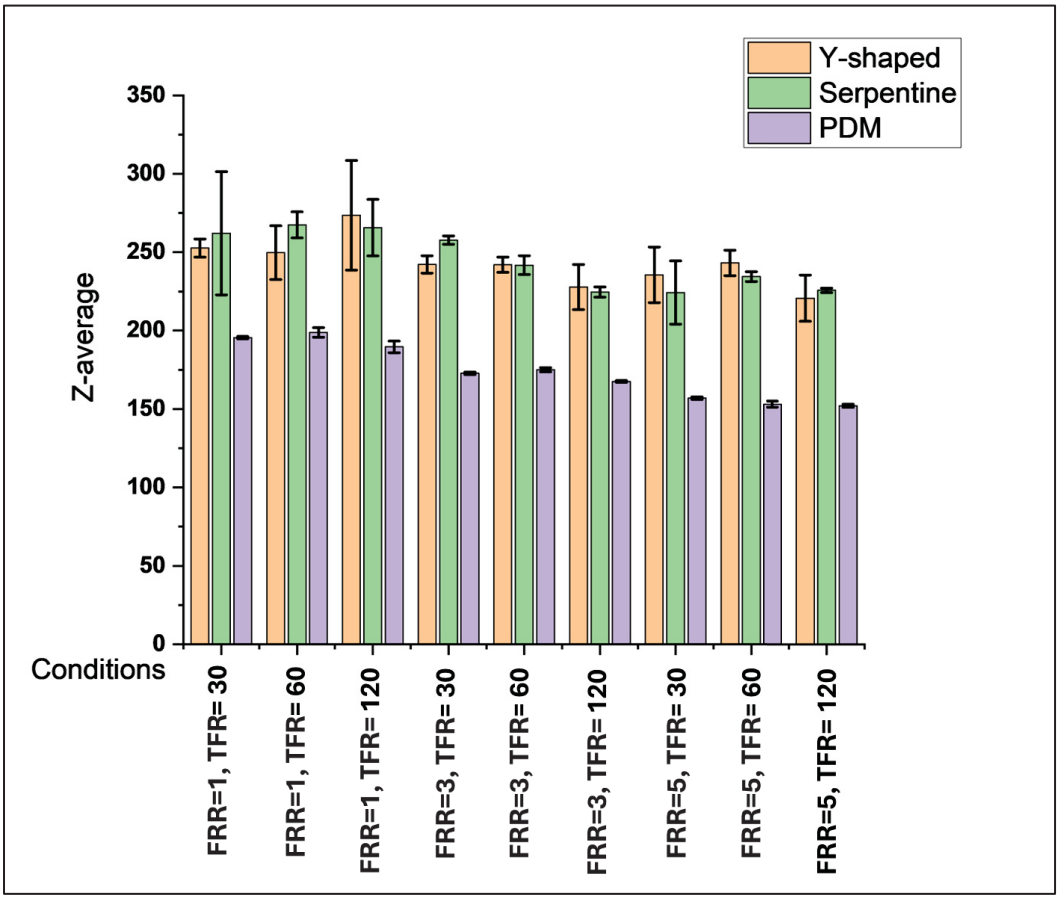


Figure 4.1 Results of characterization of the liposomes' size synthesized using three different milli mixers: Y-shaped, Serpentine and PDM with error bars

As in Figure 4.1, the Z-average of all the samples were almost below 300 nm. In this chart, orange color represents Z-average of the samples produced using Y-shaped milli mixer, green color represents samples produced by Serpentine milli mixer and purple color represents samples produced by PDM milli mixer. There is an error bar for each sample indicating the variance of liposomes' Z-average. The conditions under which the samples were produced are indicated under the bars. For example, under the condition of FRR equaling 1 and TFR equaling 30, three samples were synthesized using each of the milli mixers. As we can observe

in the results of all samples, there is a consistent trend of decreasing liposome Z-average with increasing TFR and FRR. To facilitate comparing of results, the following charts were created.

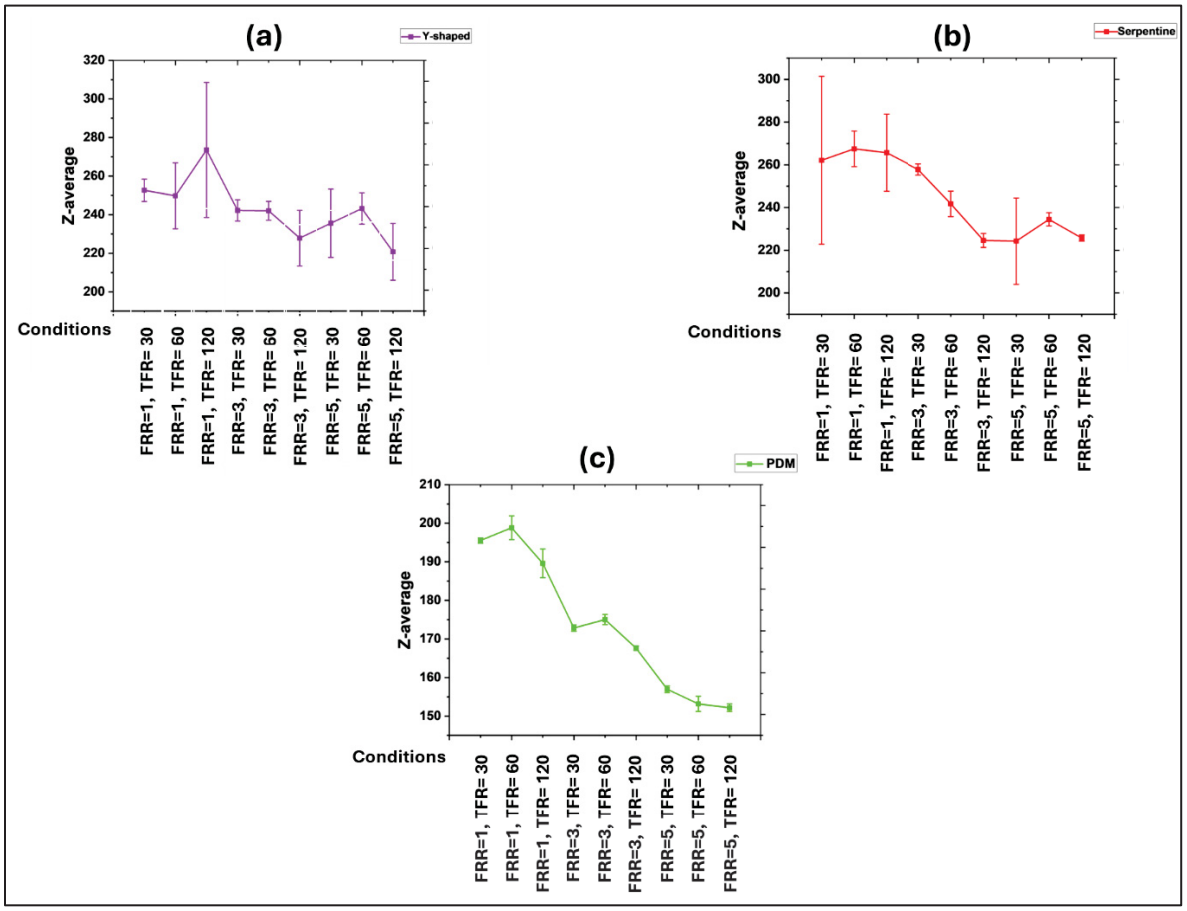


Figure 4.2 Comparison of the results of liposomes Z-average synthesized in three different milli mixers; Y-shaped, Serpentine, and PDM milli mixers

As in Figure 4.2, there is a consistent trend of decreasing liposome size by increasing the FRR value from 1 to 5 when keeping TFR values constant. This finding suggests that changing the values of the FRR can effectively control liposomes Z-average. This trend of reduction of liposome size by increasing FRR values could be related to the effect of flow rates and the velocity of the fluid on the mixing phenomenon and liposome dispersion within the process of mixing. In another word, by increasing FRR values, the dispersion of the lipid component in aqueous phase increases which results in more effective mixing and forming smaller liposomes respectively. This phenomenon also could be explained by the shear forces inside the mixing,

where by increasing the flow rate ration or FRR values, shear forces increase that results in higher lipids dispersion and smaller liposomes size. The effect of shear forces is even more obvious in the results of Z-average of the liposomes produced in PDM milli mixer, where the Z-average of the samples produced in PDM milli mixer is lower than the Z-average of the liposomes produced in Y-shaped and Serpentine milli mixers because of the narrower design of the mixing channel of the PDM milli mixer provides stronger shear forces in comparison with the other two milli mixers.

Respectively, if we investigate the influence of increasing TFR from 30 mL/h to 120 mL/h at constant FRR values, the results indicate a trend of decreasing liposome size in most of the cases. This trend of decreasing in the size of the liposomes at higher TFR values indicates that TFR in fact, affects the centripetal forces within the mixing channels. By increasing TFR values to the levels at which Dean vortices are generated which significantly increases the ethanol dilution in the water, resulting in decreasing the timescale for the polarity change rate. In this situation, lipid intermediate rapidly assembles into liposomes and consequently produces smaller nanoparticles.

Overall, among the three milli mixers, this trend of decreasing liposome Z-average is more pronounced in the PDM milli mixer accompanied by smaller error bars. This can be because of the design, specific geometry of the mixing channel, and flow characteristics of the PDM mixer which can enhance the mixing efficiency by enhancing shear forces to form smaller liposomes. In addition to these factors, when the mixing channel of a milli mixer is narrower in the size, this can result in more precise control over the flow rates and turbulence inside the mixing channel, and facilitates rapid solvent exchange which increase mixing efficiency (Vreeland, 2024).

4.2 Effect of different FRRs and TFRs on liposomes' Poly Dispersity Index (PDI)

Investigating the influence of Total Flow Rates (TFRs) and Flow Rate Ratios (FRRs) on the liposomes physicochemical properties is essential for optimizing synthesis conditions. FRR

can impact how effectively the solvents are mixing inside the mixing channel, which can affect the liposomes' Polydispersity Index (PDI). On the other hand, TFR determines fluid movement and the flow dynamics within the mixing channel, which is considered another important factor in controlling PDI. Therefore, it is essential to study FRR and TFR and their effects on PDI of liposomes which are determining factors for liposomes in drug delivery applications. Figure 4.3 provides a comparative insight to the results of PDI of the liposomes synthesized in three milli mixers; Y-shaped, Serpentine and PDM.

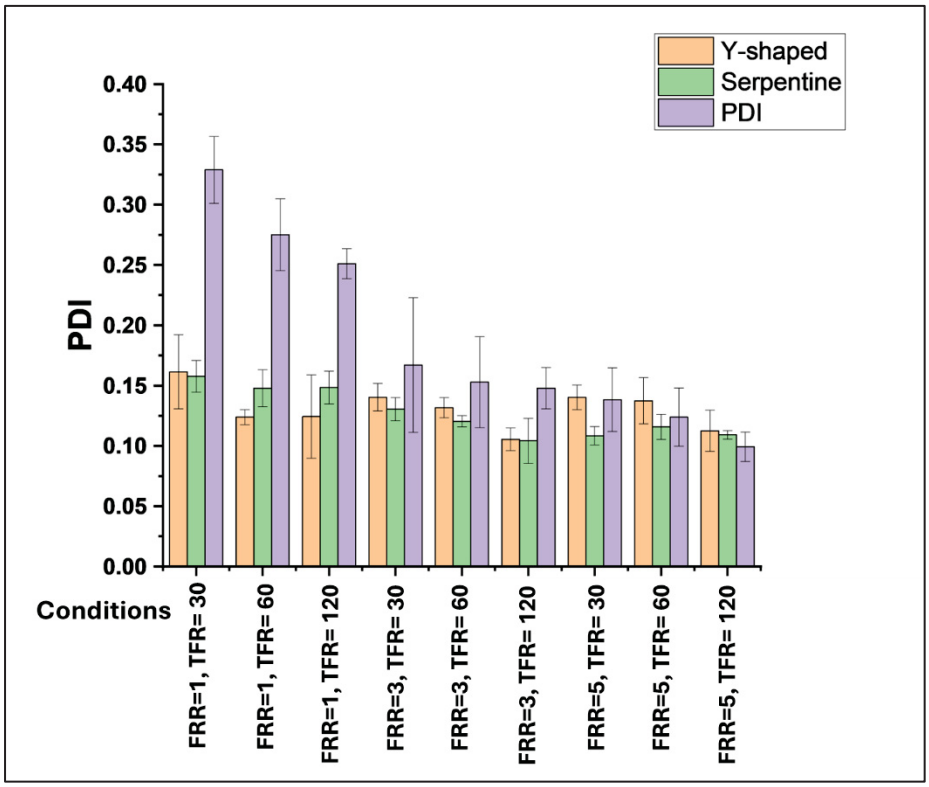


Figure 4.3 Results of characterization of the liposomes' PDI synthesized using three different milli mixers: Y-shaped, Serpentine and PDM with error bars

Figure 4.3 displays the PDI of all the samples accompanied with their error bars indicating the variance of liposomes' PDI. As we can see by changing the conditions of the synthesis there is a trend of decreasing in the liposome PDI. This trend is more obvious in the results of PDM milli mixer. To facilitate comparing and observing the results, the following charts were created.

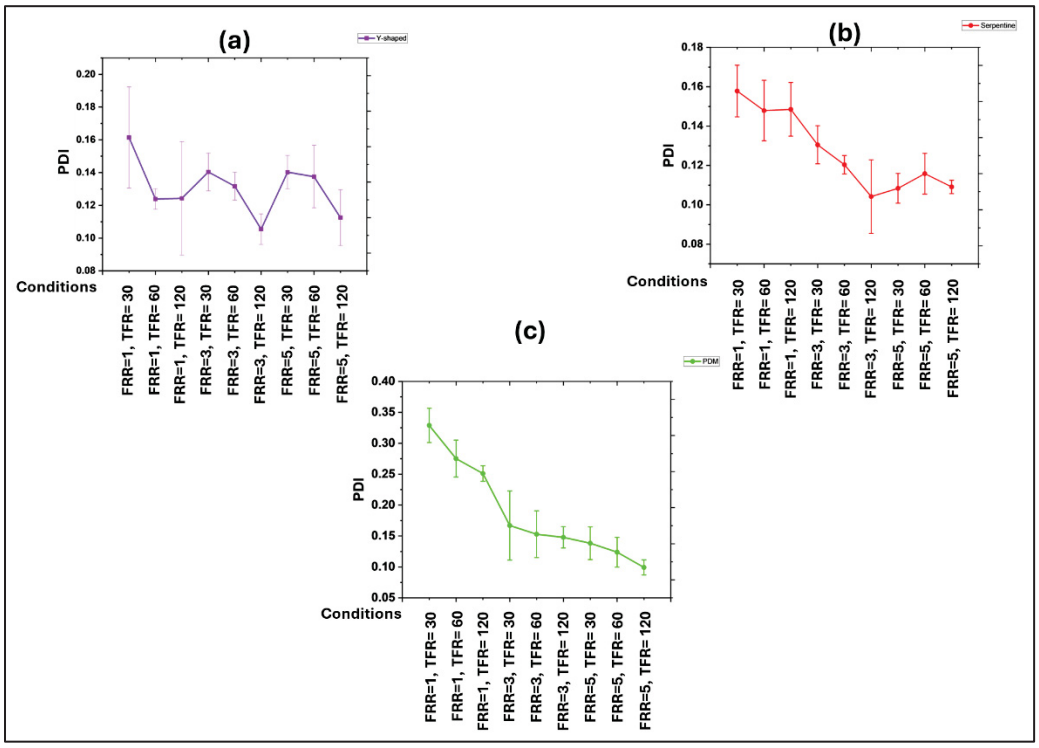


Figure 4.4 Comparison of the results of liposomes PDI synthesized in three different milli mixers: Y-shaped, Serpentine, and PDM milli mixers

As we can see in Figure 4.4, there is a trend for increasing TFR values from 30 mL/h to 120 mL/h at constant amounts of FRRs. By increasing TFR values, PDI or polydispersity index decreases in most cases. This finding indicates that at higher TFRs the size distribution of the sample is low indicating more homogeneous liposome component in the samples. As TFR increases, higher shear forces form in the channel, resulting in increased mixing efficiency and a more homogeneous size distribution.

Also, there is another trend of reduction in PDI values of liposomes by increasing the FRR value from 1 to 5 while keeping TFRs constant. This trend indicates that liposome PDI or size distribution is effectively controllable by increasing FRR at constant TFRs. This could be due to the effect of flow rates on shear forces indicating that at higher flow rates ratios, the dispersion of lipids in the aqueous phase is facilitated which results in faster formation of

liposomes from lipidic bilayers that will results in smaller and more homogeneous liposome populations.

As we can see, the trend of decreasing PDI by increasing FRR and TFR is more pronounced in the PDM milli mixer. This can be because of enhanced flow characteristics and increased shear forces because of the PDM narrower mixing channel and the design of the mixing channel which can elevate the mixing efficiency to form more uniform liposomes.

Overall, the PDI of the liposomes is another important factor that can influence directly liposomes' application in drug delivery. Smaller and more homogeneous liposomes population offer more efficient encapsulation and targeted drug delivery because of their higher ratio of surface area-to-volume. Controlling PDI of the liposomes is effective through optimizing variables of the synthesis (synthesis conditions) such as TFR and FRR. This way it is possible to synthesize liposomes with desired size and PDI for specific drug delivery needs.

4.3 Effect of different FRRs and TFRs on liposomes' zeta potential

Zeta potential is another essential parameter in liposome physiochemical characteristic that highly effects on liposomes' stability and their surface charge in liposomal suspension, which directly impacts on their interactions in human body. Therefore, it is necessary to investigate the variables controlling zeta potential in the synthesis process. In this section we focus on shedding light on the relationship between different FFRs and TFRs on zeta potential of the liposomes synthesized in three different milli mixers. This analysis will provide insights for future works in the domain of drug delivery system for liposome-based carriers by elevating our understanding of the factors affecting zeta potential of liposomal system.

Aiming to investigate the effect of these variables on the zeta potential of liposomes, three different amounts for FRRs; 1, 3, 5 and respectively TFRs; 30, 60, 120 were chosen as described before. All the 81 liposome samples were synthesized under 25℃ and undergone

characterization of zeta potential using NTA approach. The results of these samples are displayed in Figure 4.5.

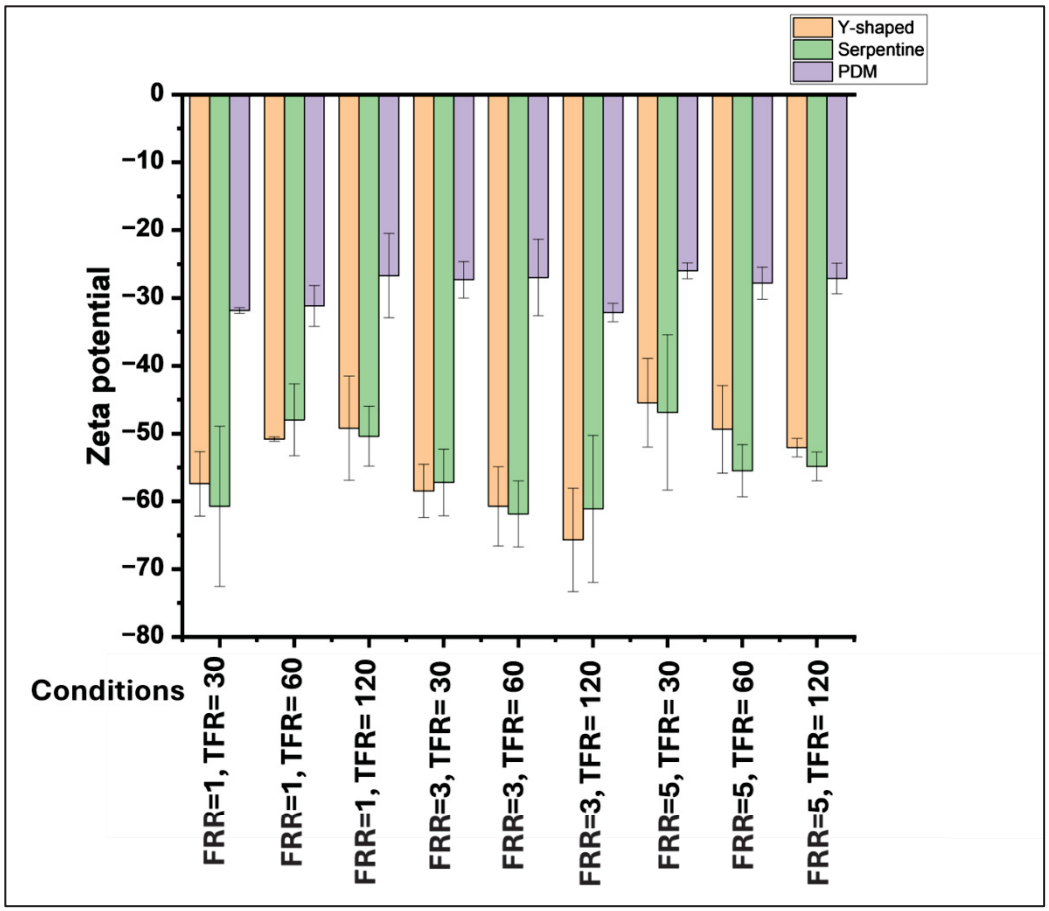


Figure 4.5 Results of characterization of zeta potential of liposomes synthesized using three different milli mixers: (a) Y-shaped, (b) Serpentine, and (c) PDM milli mixers accompanied with their error bars

As in Figure 4.5, the Zeta potential of the samples produced using three different milli mixers accompanied with their error bars are displayed. As we can see by changing the synthesis variables there is not a consistent trend of decreasing or increasing in the liposome Zeta potential obtained. In the following Figure, we can investigate easier the results of liposome characterization to find the relationship between flow rate conditions and zeta potential of the samples.

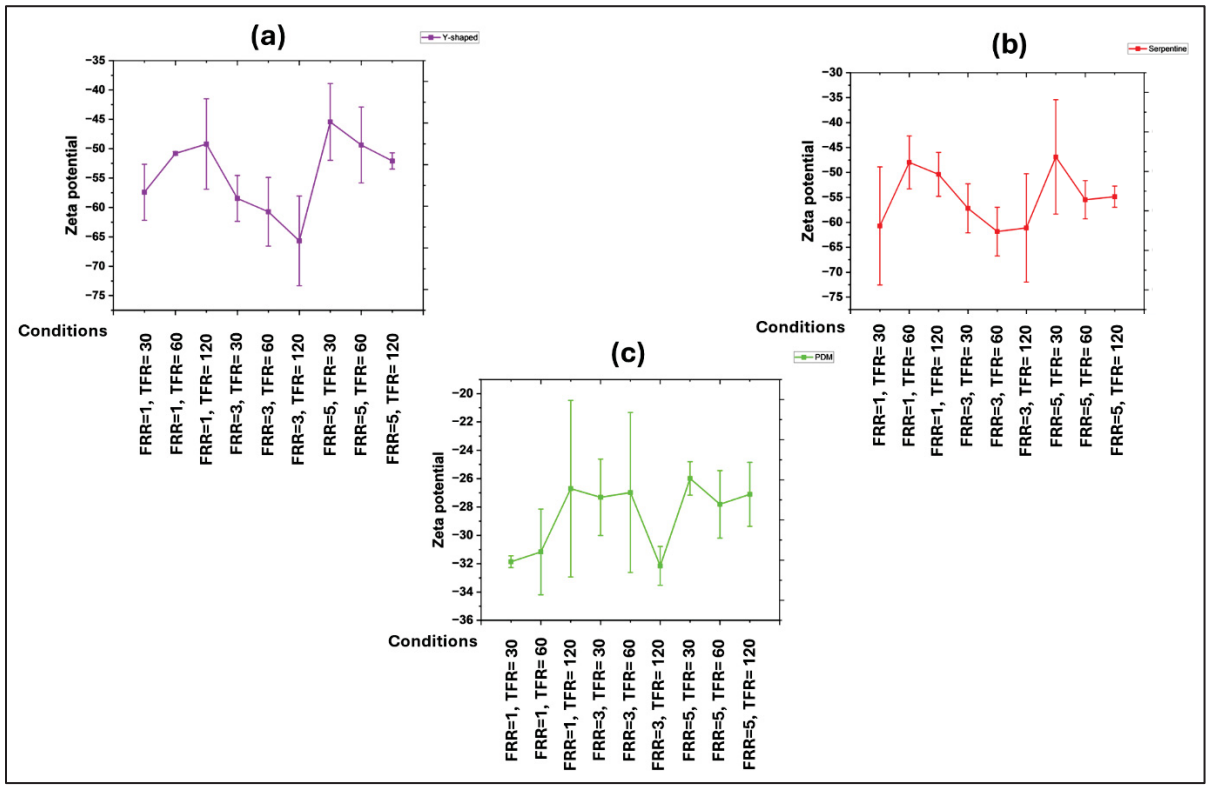


Figure 4.6 Comparison of the results of liposomes zeta potential synthesized in three different milli mixers: Y-shaped, Serpentine, and PDM milli mixers

As Figure 4.6 illustrates, by increasing the amounts of TFR from 30 to 120 mL/h at constant FRR values, there is no specific pattern found showing decreasing or increasing of the zeta potential values. For example, in Y-shaped milli mixer, if we keep FRR constant and equals 1, by increasing TFR from 30 to 120 mL/h, zeta potential shows fewer negative values while if we keep FRR constant and equals 3, by increasing TFR from 30 to 120 mL/h, zeta potential shows more negative vales. Therefore, the zeta potential values are independent of TFR values.

The same trend was observed when the amounts of TFR is constant and FRR is increasing from 1 to 5. For example, if we keep TFR constant and equals to 30 mL/h in Y-shaped milli mixer, by increasing FRR from 1 to 5, zeta potential shows fewer negative values where if we keep TFR constant and equals to 60 mL/h and increase FRR from 1 to 5, zeta potential shows more negative values when changing FRR values from 1 to 3 and it shows fewer negative

charge when changing FRR values from 3 to 5. This observation indicates that there is no constant relationship between TFR and zeta potential.

Overall, based on the results demonstrated in this section, there is no common trend of increasing or decreasing Zeta potential observed by changing TFR and FRR values, which indicates that changes of zeta potential are independent of changing the TFR and FRR values. These results highlight the importance of finding the factor(s) influencing zeta potential of the liposomes in order to synthesis stable and efficient liposomes for large-scale pharmaceutical applications.

4.4 Effect of PBS on Liposomes' physiochemical characteristics

Phosphate Buffered Saline (PBS) is a buffer solution in biological research which consists of sodium chloride (NaCl), sodium phosphate (Na_2HPO_4), and potassium phosphate (KH_2PO_4), sometimes containing potassium chloride (KCl), maintaining a stable pH of around 7.4. PBS can maintain physiological conditions that mimic human body condition for cellular survival. The reason why PBS is ideal for cell culture, molecular biology, immunology, and biochemistry applications is the fact that it can maintain the proteins and other biomolecules' biological activity.

In the synthesis of liposomes, PBS is a common solvent to suspend liposomes due to its ability to mimic human bodies' environment. Using PBS in the synthesis process affects Z-average and zeta potential of the final product because of ionic content which influences the stability of liposomes and its electrostatic interactions. In this section we delve into investigating the effect of PBS in Z-average, zeta potential and PDI of the samples.

4.4.1 Effect of PBS on liposome Z-average

In this subsection the effect of PBS on Z-average or average size of the liposome is investigated by changing the concentrations of PBS from 4% to 20% in liposomes synthesis and observing the trend of change in the liposome size. Because of the presence of the ions inside the PBS

solvent, it is expected that PBS enhance the hydrophobicity of the lipids which results in promoting better formation of liposomes and stability of the final product. In Figure 4.7 the effect of PBS on Z-average is displayed and further investigated.

It is essential to illustrate that 9 samples were synthesized for each milli mixer (Y-shaped, Serpentine and PDM), varying the TFR and FRR parameters. Each of these 9 samples was synthesized 3 times with the same conditions, resulting in a total of 27 samples per milli mixer for the reliability of the results. For example, in Figure 4.7, each chart displays 27 samples, where Y1, Y1', and Y1'' represent the three replicates of the sample Y1. This approach was employed to ensure accuracy and consistency in experimental results.

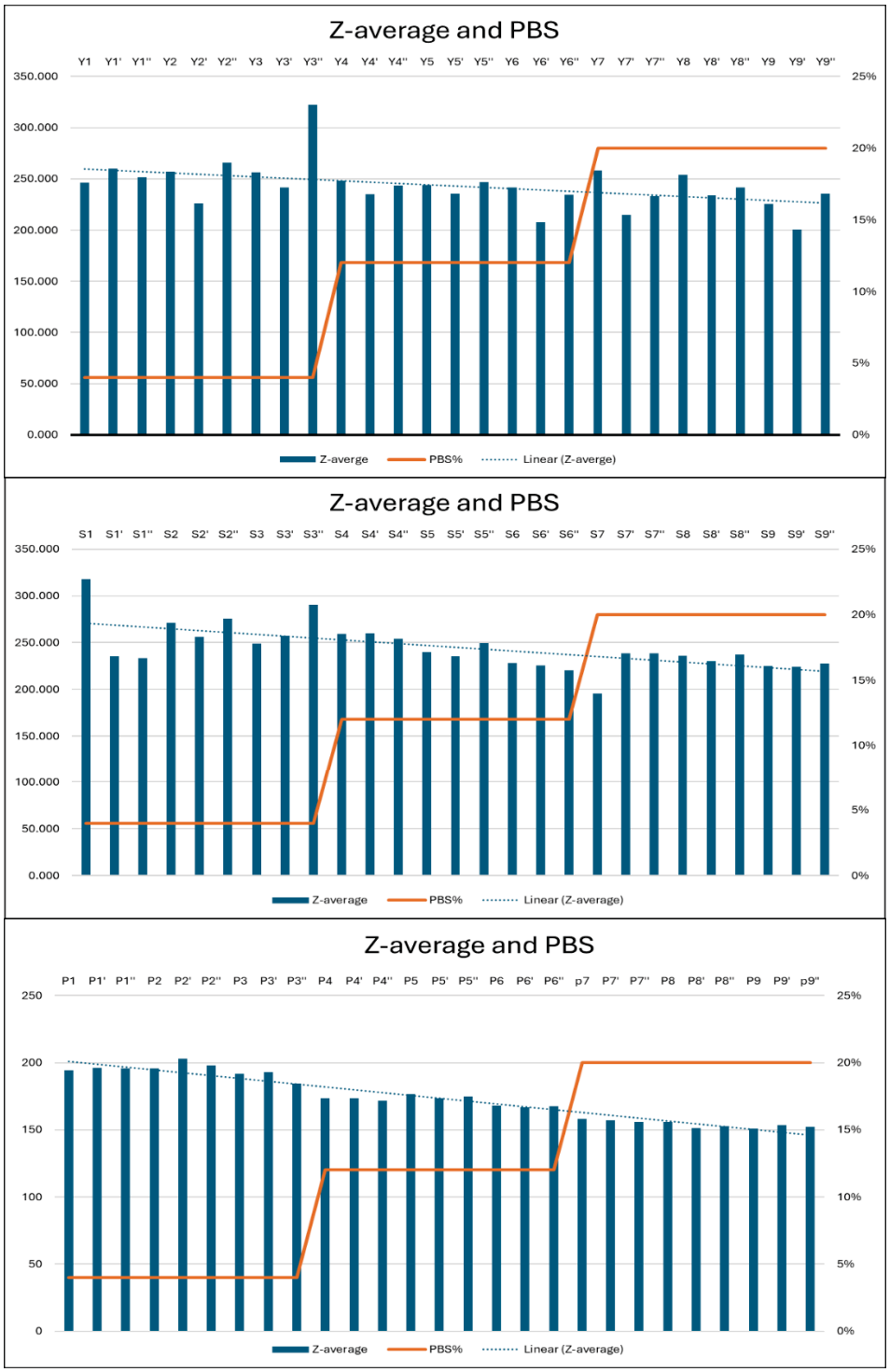


Figure 4.7 Results of characterization of Z-average of liposomes and effect of PBS used in liposome synthesis in three different milli mixers; top chart: Y-shaped, middle chart: Serpentine, and bottom chart: PDM milli mixers

As displayed in Figure 4.7, in all the samples synthesized using different milli mixers, there is a clear trend of decreasing Z-average of the liposomes by increasing the concentration of PBS in the sample. This trend is consistent in all over the experiment with different amounts of FRRs and TFRs. As we can see from left to right, low concentration of PBS (PBS=4%) resulted in larger liposomes with higher Z-average values, whereas at higher PBS concentrations (PBS=20%), liposomes are smaller with lower Z-average value s. As an example, in the liposomes synthesized using PDM milli mixer, when the PBS is 4% the amount of liposome Z-averge is around 200 nm, while by increasing the amount of PBS to 20%, Z-average decreased to around 150 nm.

This observation can be related to the ionic strength of the PBS solvent. By increasing PBS percentage in the solvent, higher ionic strength increases the electrostatic interactions between the aqueous phase and the lipid molecules which promotes the formation and stabilization of the liposomes leading to formation of smaller liposomes.

This finding illustrates the important role of PBS in determining the size of liposomes. The ability to control the size of the liposomes by varying PBS percentages is a valuable tool to synthesize liposomes with specific size for different applications such as in drug delivery where particle size play an important role in distribution and efficiency of the encapsulated agents.

4.4.2 Effect of PBS on liposome PDI

Polydispersity index or PDI is one of the important factors which indicates the size distribution of liposomes within a sample. It is expected that the presence of PBS would significantly influence the PDI of the samples because of its ionic strength as observed for the liposome size. In Figure 4.8, three charts are provided which display the effect of PBS on the size distribution or PDI of the samples.

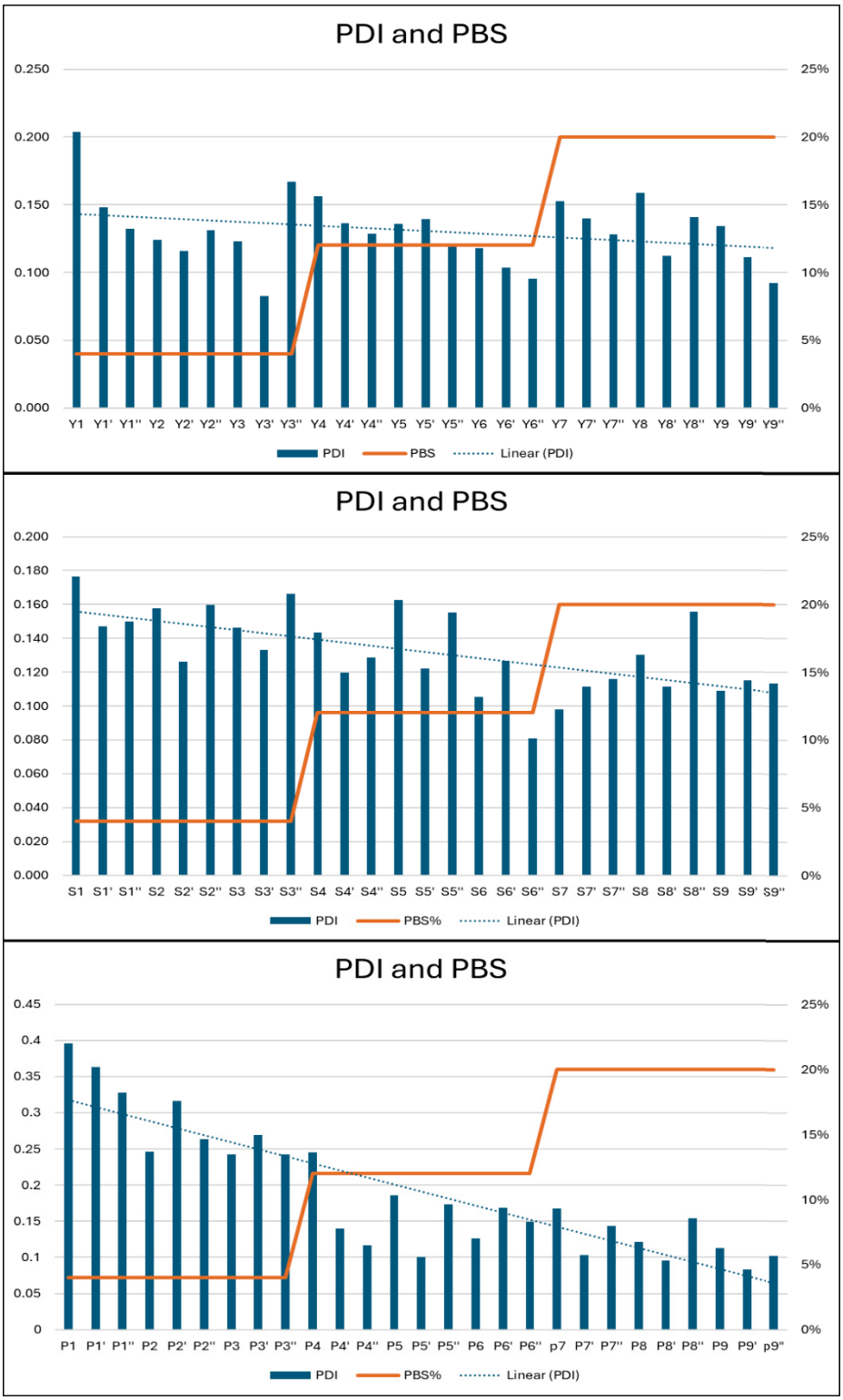


Figure 4.8 Results of characterization of PDI of liposomes and effect of PBS used in liposome synthesis in three different milli mixers; top chart: Y-shaped, middle chart: Serpentine, and bottom chart: PDM milli mixers

As in Figure 4.8, by increasing the amounts of PBS from 4% to 20% in liposome synthesis, PDI of the samples decreased. In another word, samples with higher PBS concentrations contain liposomes with lower PDI suggesting a more uniform size distribution which is one of the key elements for the application of liposomes in drug delivery domain. This observation can be explained by the presence of ions in PBS solvent enhancing the electrostatic interactions between liposomes through mixing process which can prevent aggregation of the particles. This would lead to production of more homogeneous liposomes with lower PDI.

In addition, the presence of these ions in PBS could influence mixing efficiency during the mixing process which concludes to synthesis of smaller liposomes with more homogeneous size distribution I the aqueous phase. This result is consistent with the previous studies that have been reported by Chaymaa et al. and Ruben et al. about the ionic strength on liposome size and stability (López, 2020; Zouggari, 2022). However, by decreasing PBS concentrations ionic strength decreases following by weaker electrostatic interactions that make more aggregations within the liposome sample.

4.4.3 Effect of PBS on liposome Zeta potential

In this study, we investigated the effect of PBS on zeta potential or surface charge of the liposomes. Liposome stability and compatibility is highly dependent on their surface charge that affects particle aggregation. In this subsection we would delve into investigation of the influence of PBS on zeta potential of liposomes. in Figure 4.9 the results of zeta potential experiments which are obtained from 81 samples produced using three different milli mixers are provided.

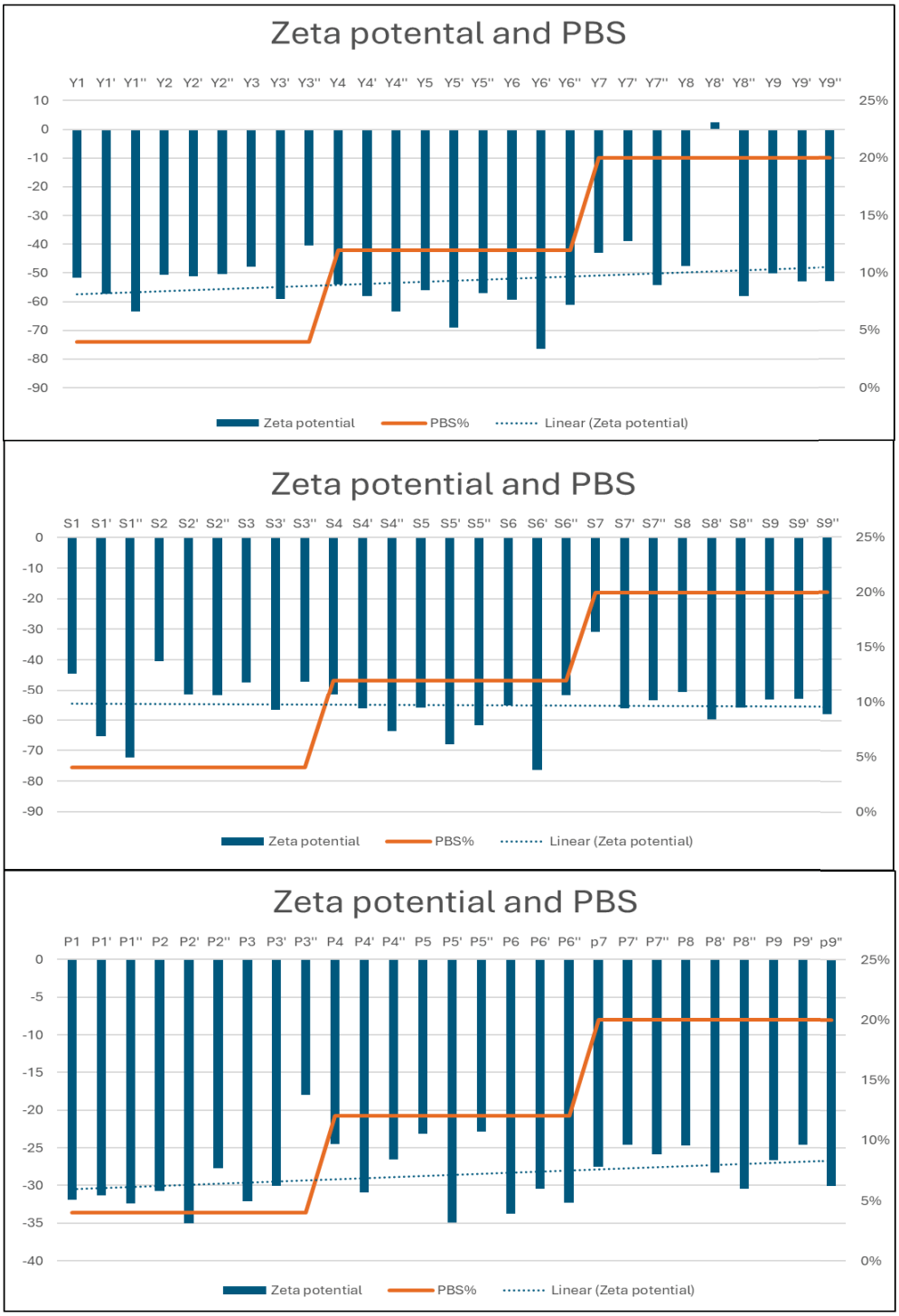


Figure 4.9 Results of characterization of zeta potential of liposomes and the effect of PBS used in liposome synthesis in three different milli mixers; top chart: Y-shaped, middle chart: Serpentine, and bottom chart: PDM milli mixers

As in Figure 4.9, the data illustrate that the concentration of PBS is affecting the zeta potential of the liposomes. This influence is more obvious in the samples synthesized using Y-shaped and PDM milli mixers. By increasing PBS concentrations from 4% to 20%, the negativity of the zeta potential decreased indicating higher PBS concentrations lead to less negative zeta potential values. In fact, PBS is a buffer with a pH equaling 7.4. At lower concentrations, for example 4%, the buffering capacity offered by PBS is weaker and the solution is more prone to changes in pH values because of the liposome environment and external factors. But as PBS concentration increased to 20%, the pH stabilized around neutral that reduces negative charge on the surface of the liposomes. This way, increasing PBS concentration can decrease the negativity of the zeta potential of liposomes.

As a summery Z-average, PDI and zeta potential of the liposomes are highly affected by PBS concentrations. By increasing PBS concentrations and promoting ionic strength, liposomes with smaller size lower size distributions and lower negative zeta potential values were produced because of the effect of PBS on altering and stabilizing pH of the solution and elevated mixing efficiency. Understanding these effects is necessary for synthesizing of liposomes and optimizing this process for various biomedical applications.

4.5 Chapter 4 Conclusions

In this chapter a parametric investigation of critical variables which can control physicochemical properties of liposome is performed. Based on the investigation liposome size was basically controlled by FRR and TFR to some extent by PBS concentration. TFR and PBS concentrations were two important variants in controlling PDI of liposomes where FRR had limited influence on size distribution of liposomes. Finally, for controlling zeta potential, FRR and PBS concentrations play an important role.

The results displayed that the Serpentine and specially PDM milli mixers can produce smaller liposomes at higher FRRs and TFRs, without increasing the PDI of the liposome population.

Maintaining low polydispersity in small liposomes is an advantage for passive targeting of organs and tissues with high specificity in drug delivery application.

Understanding these variables and factors is crucial for optimizing liposome synthesis to obtain liposomes with physiochemical characteristics suitable for pharmaceutical and industrial scale production using scaled-up milli mixers.

CHAPTER 5

COMPARISON OF THE RESULTS OF LIPOSOMES PRODUCED BY MICROMIXERS VERSES THE ONES PRODUCED BY SCALED-UP MILLI MIXERS: RESULTS AND DISCUSSION

The aim of this chapter is to investigate the impact of scaling up the micromixer to a milli mixer on the physiochemical characteristics of the final liposomes by comparing their results. In this work a scaled-up PDM milli mixer was used to synthesis liposomes and the liposomes were characterized using Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) principles and compared their characteristics with those obtained by López using a PDM micromixer. The focus of this comparison is basically on key parameters such as polydispersity index (PDI), liposome size or Z-average, and zeta potential, to evaluate how increasing the cross-sectional dimensions of the channels of a micromixer can influence the final liposome characteristics. This comparative analysis is essential in estimating the effectiveness and feasibility of using milli mixers for industrial scale liposome production. It also can provide an insight into any potential differences in uniformity, stability, biocompatibility and overall quality of the liposomes produced using the scaled-up structures.

This section investigates the comparative analysis of liposomes synthesized using a10 times scaled-up PDM milli mixer and the PDM micromixer designed by Lopez (López, 2020). In this evaluation, key parameters such as liposome size, polydispersity index (PDI), and zeta potential are investigated. The effect of total flow rates (TFR) and flow rate ratios (FRR) on these parameters are further investigated.

Run Order	Factors		Responses		
	FRR	TFR (mL/h)	Z-Average (nm)	PDI	Zeta Potential (mV)
1	6.5 (0)	3.0 (−1.41)	133.50	0.185	−31.6
2	1.0 (−1.41)	10.5 (0)	190.70	0.060	−38.8
3	10.4 (1)	15.8 (1)	67.52	0.202	−23.1
4	6.5 (0)	18.0 (1.41)	66.63	0.185	−29.8
5	12.0 (1.41)	10.5 (0)	75.09	0.232	−37.9
6	10.4 (1)	5.2 (−1)	133.5	0.174	−32.1
7	2.6 (−1)	5.2 (−1)	119.40	0.223	−35.2
8	2.6 (−1)	15.8 (1)	86.48	0.217	−29.9
9	6.5 (0)	10.5 (0)	81.81	0.207	−32.3
10	6.5 (0)	3.0 (−1.41)	120.70	0.179	−24.6
11	1.0 (−1.41)	10.5 (0)	197.00	0.072	−28.5
12	10.4 (1)	15.8 (1)	62.10	0.270	−28.8
13	10.4 (1)	5.2 (−1)	120.20	0.170	−33.9
14	6.5 (0)	18.0 (1.41)	57.14	0.238	−33.8
15	12.0 (1.41)	10.5 (0)	74.14	0.245	−30.7
16	2.6 (−1)	5.2 (−1)	122.4	0.207	−31.5
17	2.6 (−1)	15.8 (1)	88.74	0.221	−36.3
18	6.5 (0)	10.5 (0)	72.23	0.230	−37.7
19	6.5 (0)	10.5 (0)	73.81	0.235	−27.6
20	6.5 (0)	3.0 (−1.41)	116.00	0.189	−26.9
21	1.0 (−1.41)	10.5 (0)	199.70	0.064	−29.6
22	10.4 (1)	15.8 (1)	52.71	0.228	−28.2
23	10.4 (1)	5.2 (−1)	110.4.	0.184	−37.7
24	6.5 (0)	18.0 (1.41)	52.14	0.265	−34.4
25	12.0 (1.41)	10.5 (0)	73.80	0.247	32.4
26	2.6 (−1)	5.2 (−1)	131.60	0.206	−36.5
27	2.6 (−1)	15.8 (1)	90.27	0.241	−30.2
28	6.5 (0)	10.5 (0)	77.18	0.223	−27.6
29	6.5 (0)	10.5 (0)	77.24	0.262	−30.1

Figure 5.1 Results of synthesis of liposomes synthesized by Lopez et al., taken from (López, 2020)

In Figure 5.1, the physicochemical characteristics of liposomes such as size (Z-average), Polydispersity Index (PDI), and zeta potential are shown. The factors affecting liposome psychochemical characteristics including Total Flow Rate (TFR) and Flow Rate Ratio (FRR) are indicated. According to the results obtained from López's liposomes synthesis using the PDM micrometer mixer, we can investigate the effect of increasing the cross-sectional dimensions of the mixing channel from micrometer in micromixers to millimeter in milli mixers on the final liposome characteristics. The following subsections will delve deeper into this impact.

5.1 Liposomes Size Comparison

Liposome size formulations using in the industry is ranging between 50-200nm (Huwyler, 2018) and the goal of this study is to synthesize liposomes in this size range using milli mixers. In this project, we kept the temperature constant at 40 ℃ and used three different FRRs (Flow Rate Ratios) and three different TFRs (Total flow rates) to analyze the effect of these variables on liposome characteristics. In Appendix-A I-3, the information related to size, TFR, and FRR of the liposomes synthesized using milli mixers is provided.

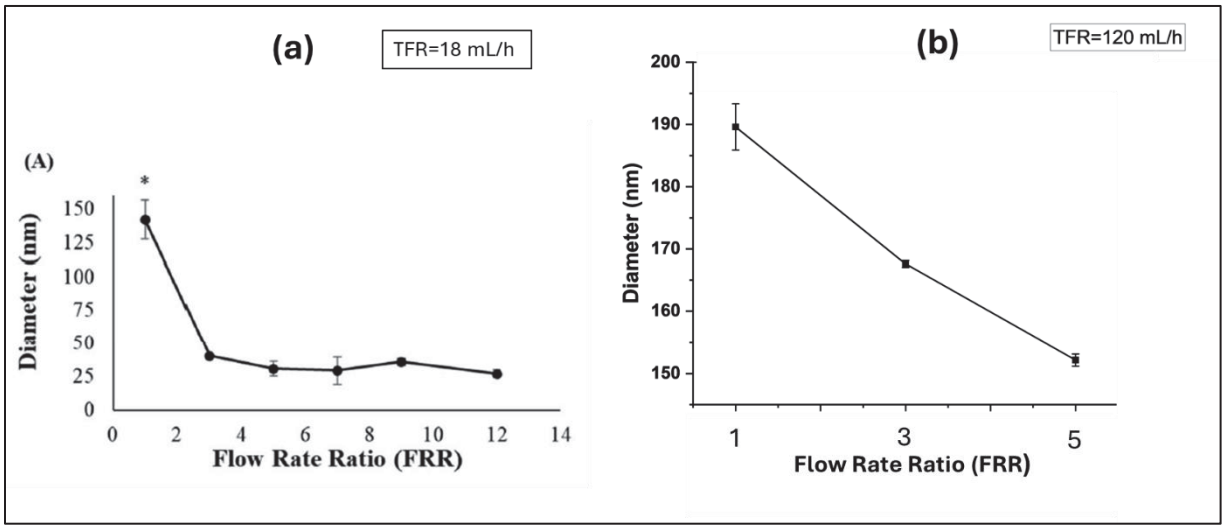


Figure 5.2 Diameter of the liposomes synthesized using PDM (a) micromixer Taken from (López, 2020) and (b) milli mixer from this study

Figure 5.2 displays two charts investigating the effect of different values of FRR on diameter of the liposomes. In chart (a), the synthesis condition includes constant TFR equals 18 mL/h with six different FRR values at 40℃ and the synthesis has been done in a PDM micromixer while in chart b, we have constant TFR equals 120 mL/h with three different FRR values at 40℃ and the synthesis has been done in a PDM milli mixer.

The results of the liposome size indicate that the sizes ranged from 151.1 nm to 189.6 nm in chart (b), which remains within the size range suitable for industrial scale applications. As we can observe, increasing FRR values at constant TFR leads to a reduction in the diameter of the liposomes. In chart b, by increasing FRR values at constant TFR the size is decreasing from 142 nm to 40 nm. Although there is a difference between the diameter of the liposomes in chart (a) and (b) because of the synthesis temperature (with increasing temperature resulting in smaller liposomes), both charts exhibit a consistent trend of reduction in liposome size by increasing FRR values at constant TFR (López, 2020). This comparison and consistent trend demonstrate the efficacy of the PDM milli mixer.

Flow Conditions		DLS			
FRR	TFR (mL/h)	Z-Average (nm)	Mean Size by Number	PDI	SD by Number (nm)
1	18	199.3	149.00	0.063	43.83
9	18	65.47	37.61	0.247	11.28

Figure 5.3 Results of liposome size and PDI characterization taken from (López, 2020)

Figure 5.3, based on the results reported by Ruben, at FRR=1, TFR=18 mL/h, a Z-average of 199.3 nm was reported as the highest average size and at FRR=9, TFR=18 mL/h, an average size of 65.47 nm. The liposome sizes are generally smaller in the liposomes synthesizes using micromixers in comparison with the ones synthesized using milli mixers, and this can be because of various possible reasons such as: higher synthesis temperature, and narrower mixing channel which can increase mixing efficiency by higher control over the flow rates inside the mixing channel and turbulence inside the mixing channel and facilitates rapid solvent exchange. Overall, the observed trend in the results of both PDM milli mixer and micromixer is the same in the results of liposomes synthesized with milli mixers and liposomes indicating that by increasing FRR at a constant TFR, a reduction in liposomes size happens.

5.2 Poly Dispersity Index (PDI) of Liposomes Comparison

Size distribution or PDI indicates the range and frequency of liposome sizes within a sample. This characteristic is crucial for liposomes application in drug delivery because it provides insight into the uniformity of the liposome population which impacts liposomes behavior in biological systems including biodistribution, circulation time, and cellular uptake especially in drug delivery applications should be low to be recognized as monodispersed. Liposome size distributions with PDI < 0.1, and PDI < 0.2 indicates low polydispersity or monodisperse populations which is suitable for pharmaceutical applications (Hood & DeVoe, 2015)

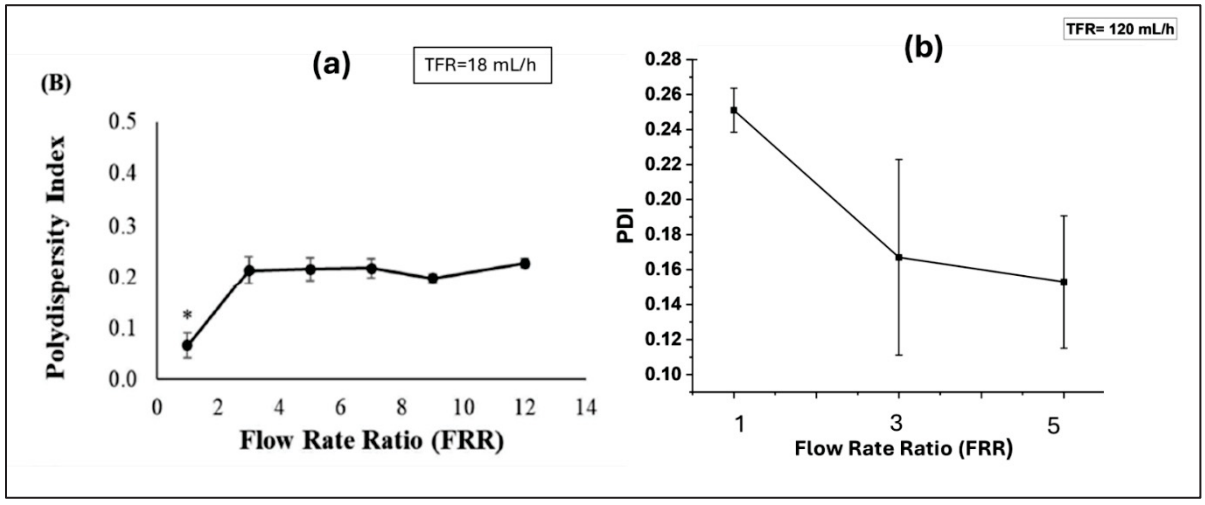


Figure 5.4 Poly Dispersity Index of the liposomes synthesized using PDM (a) micromixer Taken from (López, 2020) and (b) milli mixer from this study

Figure 5.4 displays two charts investigating and comparing the influence of different FRR values on PDI of the liposomes. Chart (a) shows the results of PDI of the liposomes synthesized in a PDM micromixer with constant TFR value equals 18 mL/h with six different FRR values at 70°C. Chart (b) displays the results of PDI of the liposomes synthesized in a PDM milli mixer with constant TFR value equals 120 mL/h with three different FRR values at 25°C.

The results of PDI in chart a indicate that by increasing FRR values at constant TFR, PDI of liposomes has been increased from 0.05 to around 0.24. Conversely, by increasing FRR at constant TFR in chart b, PDI values have been decreased from 0.28 to 0.16 indicating that by increasing FRR values at constant TFR values, we can achieve low polydispersity or monodisperse liposome population which is suitable for drug delivery and vaccine applications.

If we take a look at Appendix-A I-4, all of the results of PDI investigation in PDM milli mixer have been presented. PDI values in this experiment ranged from 0.08315 to 0.3957. The highest PDI values are obtained when FRR=1 and the TFRs is at the lowest values whereas by increasing the FRR to 3 and respectively to 5 and increasing TFR to 60 and 120 mL/h, the PDI value decreased which indicates that the most homogenous or monodispersed populations can be obtained at higher FRR and TFRs. On the other hand, Figure 5.3 displays the results from experiments of Lopez indicating that at FRR=1 and TFR=18 mL/h, the PDI is 0.063 while by increasing FRR to 9 at constant TFR, the PDI value is increased to 0.247. If we look at Figure 5.1, the same trend is prevalent in the samples.

In general, PDI values in the liposome synthesized using PDM milli mixer are higher in comparison to Lopez's work indicating that the samples are more polydisperse when they are produced in a scaled up milli mixer. This might be due to the different manufacturing techniques and materials that have been used in fabricating the structure. In Lopez's micromixer, soft lithography technique was used to fabricate the micromixer. This method includes a few steps such as photolithography for making the master mold following by forming the microchannels through casting polydimethylsiloxane (PDMS). This technic offers smoother with higher resolution surfaces inside the channel which is a crucial factor for applications requiring precise fluid dynamics a perfect feature. On the other hand, the structures manufactured using 3D printers are more durable and allow faster prototyping

compared to the PDMS ones but the possible imperfections inside the channel can affect the results of phytochemical characteristics such as PDI.

5.3 Zeta Potential of Liposomes Comparison

Zeta potential is a crucial parameter in the liposome characterization. Zeta potential is capable of providing information about the stability and surface charge of the vesicles which are important factors in their application in drug delivery and industry. This factor is calculated by measuring the difference of the potential between the stationary layer and the dispersion medium of fluid attached to the dispersed particle.

In the following, the results of the zeta potential of liposomes synthesized using PDM micromixer by Lopez and the liposomes synthesized using scaled up PDM milli mixer have been compared.

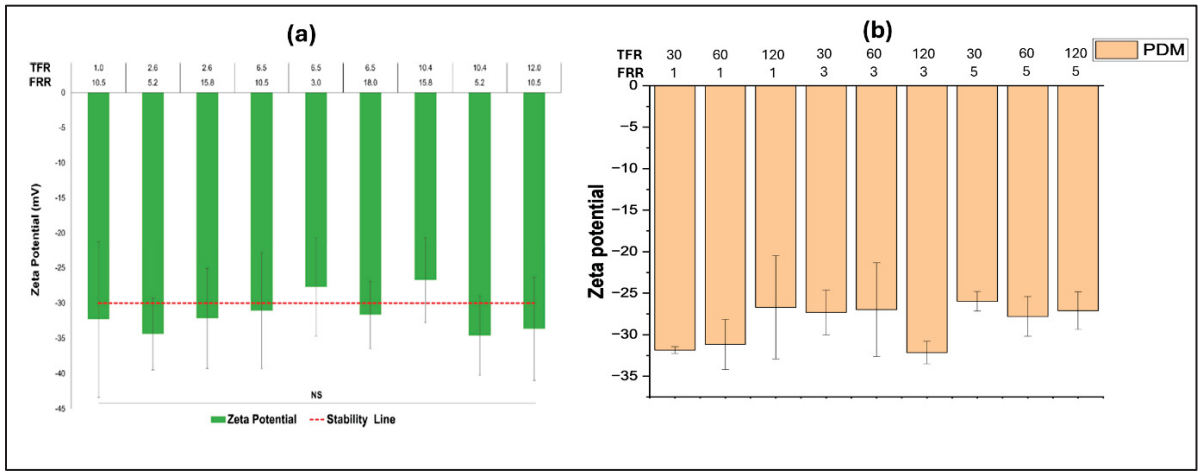


Figure 5.5 Liposome's potential synthesized; (a): using PDM micromixer taken from (López, 2020) (b): using PDM milli mixer with three different amounts of TFR and FRR at 25℃

Figure 5.5 compares the results of zeta potential of the liposomes produced using PDM (a) micromixer and (b) milli mixer. As we can explore figure (b), zeta potential ranges from -17.97 mV to -35.05 mV and the average zeta potential value is -28.72 mV in these samples. The table

of the full results is available in Appendix-A I.5. In the other hand, figure (a) displays the results of zeta potential obtained from Lopez samples. The value of zeta potential in his work ranged from -38.8 mV to -23.1 mV and the zeta potential is independent of TFR and FRR in Lopez results the same as the results obtained from liposomes synthesized using PDM milli mixer. The narrow range of liposomes' zeta potential values both in Lopez's results and the results of liposomes synthesized in milli mixer shows consistent and stable surface charge in different synthesis conditions. The more consistent and stable zeta potential values contribute to more uniform behavior and less aggregation of liposomes in biological systems.

The zeta potential value in the results of both fluidic devices is almost in the same range. The results show negative zeta potential values which contribute to the liposomes stability, biocompatibility, efficiency, and their ability in targeted delivery in drug delivery systems.

5.4 Chapter 5 conclusions

In this chapter we made a deep comparison of the trend in the results of the scaled-up PDM milli mixer with those from Lopez experiments. The comparison showed consistent results where by increasing the FRR, liposome size decreased. Also, there is a similar trend in the zeta potential results of Lopez and our results in this work, indicating that this component is not dependent to FRR and TFR. The comparison of the results indicates that both sets of experiments synthesized anionic liposomes. Anionic liposomes are liposomes with negative zeta potential values which show good stability and biocompatibility of the final product.

On the other hand, the results of the PDI of liposomes synthesized using scaled-up micromixer, showing inverse relation with increasing FRR, meaning by increasing FRR, PDI decreased while in Lopez's results, by increasing FRR, PDI has been increased. Overall, the size of the liposomes and the PDI values were higher in the scaled-up dimensions indicating that some optimization in scaling process is needed to maintain the same results as the PDM micromixer.

CONCLUSIONS

This work has focused on high reproducibility in mass production of liposomes for industrial scale while maintaining their desired physiochemical characteristic for extending liposomes in biomedical applications effectively. In this study we investigated production of liposomes using three milli mixers with different designs, including Y-shaped, Serpentine and Dean dynamic flow-based milli mixer (PDM).

We investigated the relationship between physiochemical characteristics of liposomes and mixing efficiency. The size of the liposomes synthesized using three different milli mixers ranged from 151 nm to over 300 nm, but most of the liposomes had Z-average less than 250 nm which is quite suitable for drug delivery applications.

Liposome size dispersion was almost monomodal, ranging in highly low polydisperse. This investigation shows that some of the liposomes are distributed uniformly, and the samples were almost uniform, especially the samples produced using PDM milli mixer.

Liposome zeta potential was negative in all the samples relating to the fact that they are suitable candidate for drug delivery application, because they are more stable, biocompatible, and they are basically known as long-circulating liposomes. The relationships between TFR, FRR, and PBS with liposome physiochemical characteristics such as size, PDI, and zeta potential were also analyzed. Each sample was produced three times with the same production conditions, but the liposome properties remained consistent, which demonstrated reproducibility.

Using a numerical model, which was indicating concentration dependent variables, the mixing efficiency of the mixing channels were assessed. Further parametric experiments such as fluid flow was performed to observe and qualitatively analyze the mixing phenomenon. The mixing

efficiency in PDM milli mixer was achieved faster to 90% in comparison with PDM micromixer, indicating improvement in mixing efficiency inside the mixing channel. FRR and PBS played a crucial role in altering liposome size and its zeta potential, but TFR was slightly controlling PDI and zeta potential.

In summary, this study investigated various factors in three different milli mixers, influencing liposome characteristics, and highlighted the fundamental aspects of liposome synthesis using milli mixers. This proposed method is proper for liposome production with continuous flow at high rates. Scaling up to milli mixers can improve the production rate, increase the liposome yield for industrial scale but requires further optimization on refining the design to reduce size and PDI of the liposome. This could potentially be doable by optimization of the dimensions of the mixing channel and conditions of flow to perform better at larger scale.

APPENDIX A

RESULTS OF SIZE, PDI AND ZETA POTENTIAL CHARACTERIZATIN OF LIPSOMES

The charts of Z-average (hydrodynamic diameter, nm) and PDI of the samples displayed in Chapter 4 and tables of the samples synthesized using PDM milli mixer are presented in detail in this section. As we can see in Figure-A I-1, in each milli mixer, 27 samples were synthesized but in fact we have three replicates of each synthesis condition with the same FRRs and TFRs. As an example, liposomes synthesized in Y-shaped milli mixer are labeled as Y1, Y1′, and Y1″representing replicates of the sample Y1. This means that these three samples were produced under identical conditions with the same FRRs and TFRs. In fact, we produced each sample three times so that the results would be reliable reproducible, and accurate scientifically. The sample replicates generally display results within the same range, which shows consistency in liposome synthesis process. However, some variations are found in some of the results indicating the experimental errors for minor fluctuations within the synthesis conditions.

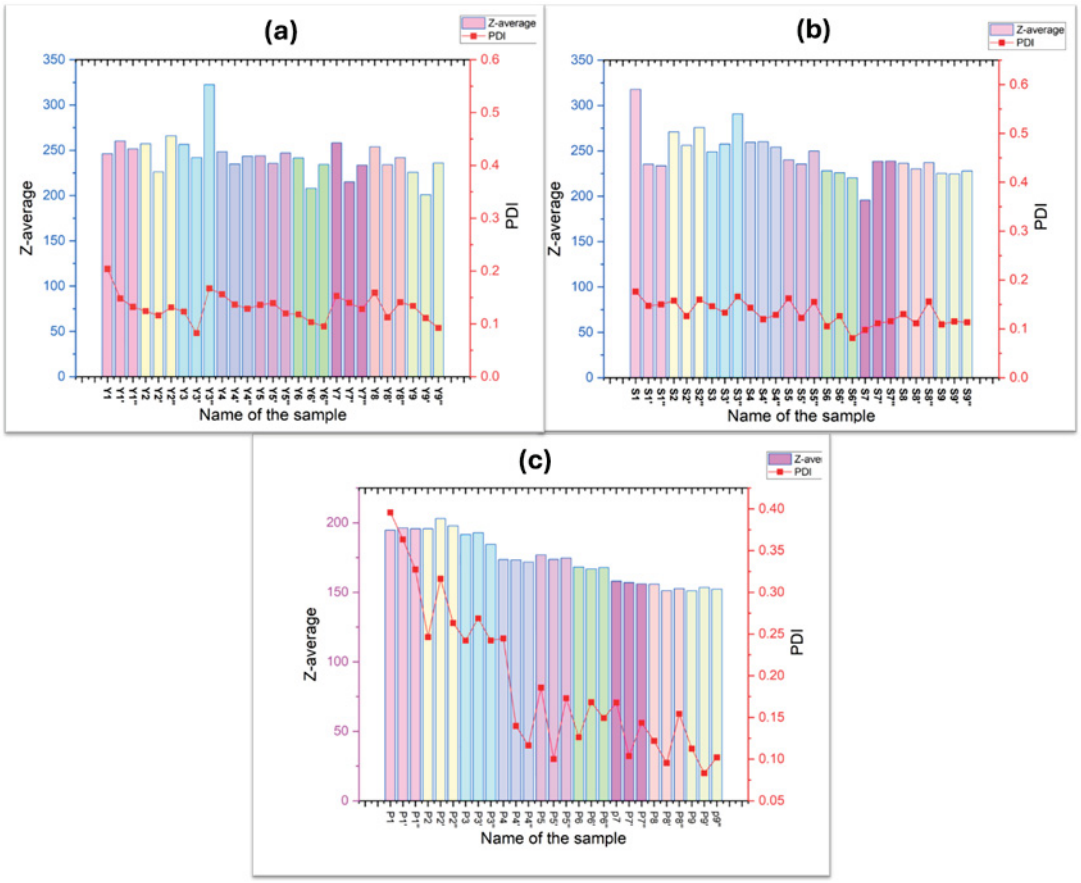


Figure-A I-1 Results of characterization of size and PDI of liposomes synthesized using three different milli mixers: (a) Y-shaped, (b) Serpentine, and (c) PDM milli mixers

In Figure-A I-1, for example in chart c, from left to right by increasing FRR and TFR, liposome size is decreased following by decreasing in PDI. This can prove that the flow rate is influencing the liposomes size and PDI. In Figure-A I-2, the results of zeta potential of 81 samples produced in three different designs of milli mixers are displayed.

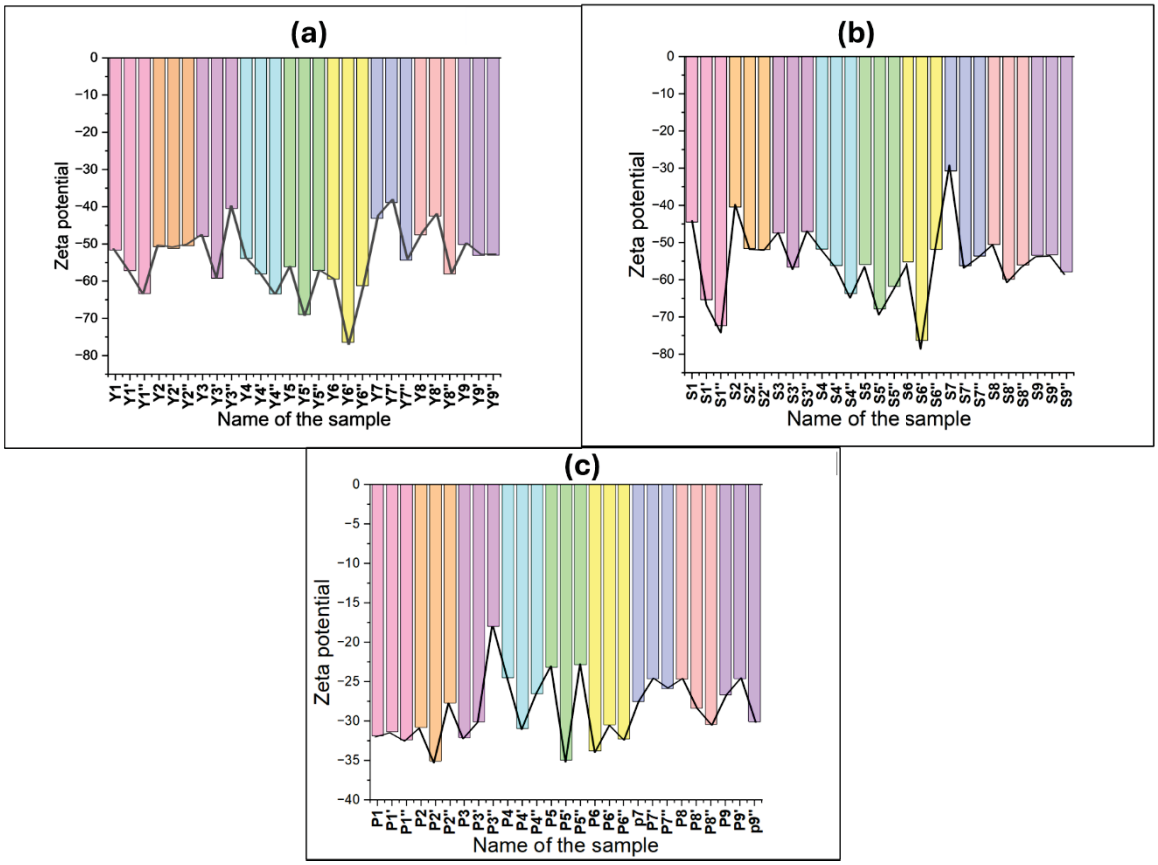


Figure-A I-2 Results of characterization of all samples' zeta potential synthesized using three different milli mixers: (a) Y-shaped, (b) Serpentine, and (c) PDM milli mixers

In Figure-A I-2, most of the results in each of the three replicates are in the same range. However, in some cases some variations are observed which could be related to either the error caused within characterization process or the experimental errors in small fluctuations of the conditions in the synthesis process.

All the information related to the samples synthesized using PDM milli mixer along with synthesis variables such as TFR and FRR and the measured z-average, PDI and zeta potential are provided in Figure-A I-3, Figure-A I-4, and Figure-A I-5.

Sample	FRR	TFR	Sample order	Temp	Z-average
PDM 1	1	30	P1	25	194.6
PDM 10	1	30	P1'	25	196.3
PDM 19	1	30	P1''	25	195.7
PDM 2	1	60	P2	25	195.7
PDM 11	1	60	P2'	25	203
PDM 20	1	60	P2''	25	197.8
PDM 3	1	120	P3	25	191.6
PDM 12	1	120	P3'	25	192.8
PDM 21	1	120	P3''	25	184.4
PDM 4	3	30	P4	25	173.5
PDM 13	3	30	P4'	25	173.3
PDM 22	3	30	P4''	25	171.7
PDM 5	3	60	P5	25	176.8
PDM 14	3	60	P5'	25	173.6
PDM 23	3	60	P5''	25	174.7
PDM 6	3	120	P6	25	168.2
PDM 15	3	120	P6'	25	166.8
PDM 24	3	120	P6''	25	167.8
PDM 7	5	30	p7	25	158
PDM 16	5	30	P7'	25	157
PDM 25	5	30	P7''	25	155.9
PDM 8	5	60	P8	25	155.8
PDM 17	5	60	P8'	25	151.1
PDM 26	5	60	P8''	25	152.6
PDM 9	5	120	P9	25	151
PDM 18	5	120	P9'	25	153.4
PDM 27	5	120	p9''	25	152.1

Figure-A I-3 Z-average or size of the liposomes synthesized using PDM milli mixer with three different amounts of TFR and FRR at 25°C

Sample	FRR	TFR	Sample order	Temp	PDI
PDM 1	1	30	P1	25	0.3957
PDM 10	1	30	P1'	25	0.2634
PDM 19	1	30	P1''	25	0.3274
PDM 2	1	60	P2	25	0.2462
PDM 11	1	60	P2'	25	0.3162
PDM 20	1	60	P2''	25	0.2631
PDM 3	1	120	P3	25	0.2421
PDM 12	1	120	P3'	25	0.2688
PDM 21	1	120	P3''	25	0.2422
PDM 4	3	30	P4	25	0.2448
PDM 13	3	30	P4'	25	0.1398
PDM 22	3	30	P4''	25	0.1164
PDM 5	3	60	P5	25	0.1857
PDM 14	3	60	P5'	25	0.1
PDM 23	3	60	P5''	25	0.173
PDM 6	3	120	P6	25	0.1262
PDM 15	3	120	P6'	25	0.1682
PDM 24	3	120	P6''	25	0.1492
PDM 7	5	30	p7	25	0.1678
PDM 16	5	30	P7'	25	0.1037
PDM 25	5	30	P7''	25	0.1435
PDM 8	5	60	P8	25	0.1219
PDM 17	5	60	P8'	25	0.09542
PDM 26	5	60	P8''	25	0.1543
PDM 9	5	120	P9	25	0.1126
PDM 18	5	120	P9'	25	0.08315
PDM 27	5	120	p9''	25	0.1021

Figure-A I-4 PDI of the liposomes synthesized using PDM milli mixer with three different amounts of TFR and FRR at 25℃

Sample	FRR	TFR	Sample order	Temp	Zeta potential
PDM 1	1	30	P1	25	-31.86
PDM 10	1	30	P1'	25	-31.35
PDM 19	1	30	P1''	25	-32.37
PDM 2	1	60	P2	25	-30.78
PDM 11	1	60	P2'	25	-35.05
PDM 20	1	60	P2''	25	-27.68
PDM 3	1	120	P3	25	-32.08
PDM 12	1	120	P3'	25	-30.06
PDM 21	1	120	P3''	25	-17.97
PDM 4	3	30	P4	25	-24.49
PDM 13	3	30	P4'	25	-30.94
PDM 22	3	30	P4''	25	-26.52
PDM 5	3	60	P5	25	-23.12
PDM 14	3	60	P5'	25	-34.96
PDM 23	3	60	P5''	25	-22.85
PDM 6	3	120	P6	25	-33.78
PDM 15	3	120	P6'	25	-30.43
PDM 24	3	120	P6''	25	-32.26
PDM 7	5	30	p7	25	-27.49
PDM 16	5	30	P7'	25	-24.62
PDM 25	5	30	P7''	25	-25.84
PDM 8	5	60	P8	25	-24.66
PDM 17	5	60	P8'	25	-28.34
PDM 26	5	60	P8''	25	-30.42
PDM 9	5	120	P9	25	-26.67
PDM 18	5	120	P9'	25	-24.58
PDM 27	5	120	p9''	25	-30.06

Figure-A I-5 Zeta potential of the liposomes synthesized using PDM milli mixer with three different amounts of TFR and FRR at 25℃

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