Microfluidics (NanoAssemblr©) as a potential platform for nanocarrier formation

Lim, Shaun Wen Zheng

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**Microfluidics (NanoAssemblr©) as a potential platform for
nanocarrier formation**

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NTU-Northwestern Institute for Nanomedicine (NNIN)

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nanocarrier formation**

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Thesis submitted to Nanyang Technological University in partial
fulfilment of the requirements for the degree of Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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10th May 2021

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Date

Assistant Professor Bertrand Czarny

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal in which I am listed as an author.

Chapter 4 and 5 is published as Microfluidic-directed self-assembly of liposomes: Role of interdigitation. Shaun W.Z. Lim*, Wong Y.S, Czarny B, Venkatraman S. Journal of Colloid and Interface Science, Volume 578, 2020, Pages 47-57.

The contributions of the co-authors are as follows:

- Prof Subbu provided the initial direction of the project and edited manuscript
- I prepared the first manuscript drafts. The manuscript was revised by Dr Wong Yee Shan and Asst Prof Bertrand.
- I co-designed the study with Dr Wong Yee Shan and performed all the laboratory work at the School of Materials Science and Engineering and NTU-Northwestern Institute of Nanomedicine Laboratory. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation. Cryo-Tem was kindly provided by Mr Melvin Liew.

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- Asst Prof Bertrand and Prof Subbu provided the initial direction of the project and edited manuscript.
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10th May 2021

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Shaun Lim Wen Zheng

Abstract

Since the inception of nanocarriers as drug delivery agents, liposomes stood out as a suitable candidate due to its non-toxic and biodegradable characteristics. Over the past five decades, much research efforts have attempted to elucidate the drug delivery potential of liposomes with some achieving clinical success. However, more work can be put towards refining drugs/bioactives loading, and fabrication methods. Herein a new technique, microfluidics, serves to revolutionise liposome fabrication method to improve the desired research outcomes. This thesis is divided into three parts, namely: investigating the role of lipid compositions and process parameters on liposomal self-assembly, understanding the mechanism of liposome formation as well as optimizing encapsulation of actives in liposomes.

Microfluidics involve precise mixing of solvents and manipulation of chip architecture to achieve changes in liposome size. In this work, the method known as mixer-assisted microfluidics was used. It involves a staggered herringbone mixer (SHM) to promote rapid and controlled mixing.

In the first part of the thesis, different types of lipid composition such as length, saturation, solvent choice and additives were explored to evaluate their effect on size and polydispersity if liposomal assembly was successful using microfluidics as compared to thin film hydration. Results showed that liposomal assembly was most successful with lipids with unsaturation in at least one hydrocarbon chain. Fully saturated lipids with lipid carbon chains more than 14 were unable to form liposomes. It was clear that the method may not be universally suited for all lipid types so varied operating conditions must be involved together with investigation towards the actual cause of this aggregation. A phenomenon known as membrane interdigitation was discovered in the first part. Interdigitation refers to the phenomenon where two opposing leaflets of a bilayer interpenetrate into one another and form a single layer. When this happens, aggregation results as the single layer is not thermodynamically stable. It was discovered that saturated double chained lipids with transition temperature (T_m) below room temperature were especially susceptible to lipid interdigitation. Strategies to prevent interdigitation is to either remove ethanol above the lipid's main transition temperature (T_m), preventing the formation of interdigitated structures and subsequent aggregated states or by the incorporation of the inhibiting additives, such as cholesterol.

In the second part of the thesis, the process parameters such as effects of flow or ratio of organic solvent on liposome formation were explored. Results showed that the higher the ratio of aqueous phase to organic solvent is, the smaller the particle size is. The same trend was shown for higher flow rates of the streams. One possible explanation is because of the shearing effect used in the mixer-assisted microfluidics. As more aqueous buffer is used, the organic layer containing the lipid has lesser time to assemble into the liposomes before being exposed to an aqueous environment, leading to a smaller size.

With the results from part 1 and 2, further exploration of microfluidics were performed. 2 model drugs, dextran FITC and latanoprost were used as hydrophilic and lipophilic model drug respectively. Results showed that unsaturated lipid, EggPC showed encapsulation of 27% for dextran FITC and 40% for latanoprost. Drug release profiles show sustained release for both types of drug. The drug loadings were similar to that produced using conventional thin film hydration method. As conventional thin film hydration are constantly plagued with reproducibility and ease of handling issues, microfluidic technology is poised to resolve these problems. The advantages of using microfluidics include ease of handling, high reproducibility, size control without involving additional size reduction steps, as well as a fully automated mixing process. With these benefits mentioned above, this shows great promise as an alternative liposome fabrication method.

Thus, the novelty of this project lies in establishing a relationship between microfluidics and the formation of liposomes. Various studies on liposomal work with microfluidics do not explicitly describe the conditions for successful liposome formation, so the work in this project serves to complete the knowledge gap by understanding how and why different lipid types are able to form liposomes.

Lay Summary

Usage of medicine is rudimentary in modern times to treat a variety of diseases. However most drugs cannot be directly consumed or injected because the body removes these foreign entities quickly. This results in most drugs having little therapeutic effects. One of the innovative ways is to incorporate these medicines in carrier systems, such as a liposome. The traits of these carrier systems are to protect the drug from the body and releasing continuously over periods of time to treat the disease. Optimization of these drug delivery systems becomes critical to improve the therapeutic effectiveness. Liposomes are suitable due to its biocompatibility, naturally occurring, and its ability to carry both hydrophilic and hydrophobic drugs makes it a versatile carrier system.

Although many decades of laboratory research have been put into liposomes, it was unable to commission industrial outputs of the liposome formulations. Some reasons include manual labour intensive, reproducibility issues stemming from batch-to-batch variation and inability to load high amounts of drugs.

Here this report presents an alternate method to replace the laborious nature of the conventional technique. One new way of making these liposomes is using a microfluidic device. The microfluidic device used here include two inlet capillaries, one mixer and an outlet. One inlet contains the organic solvent and the lipid, while the other contain an aqueous buffer for the self-assembly to take place. The mixer is specifically shaped to ensure complete mixing in a short amount of time. The advantages of using this microfluidic device are higher reproducibility, lesser steps as compared to thin film hydration, timesaving, homogeneity of liposome size. This microfluidic system is automated; hence it reduces the random errors likely caused by the human user. This increases reproducibility and reliability of the liposome batches. In addition, the parallelization of the microfluidic chip allows an easy way to increase output without sacrificing reproducibility. Reproducibility is an important criterion because different liposome sizes will elicit different responses from the body, with larger particles getting cleared faster.

Proposing a new method to produce liposomes will require validation and it will be performed here. The general parameters are split into formulation parameters, which involve the lipids, and process parameters, which involve the microfluidic devices. For formulation parameters, lipids with different physical characteristics such as chain length and saturation will be analysed. For process parameters, the flow ratio of lipid to aqueous buffers and flow rate of the

streams will be analysed. Finally, the loading of drug moieties unto the liposomes will be performed and analysed to see if they are comparable to conventional methods of liposome fabrication.

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Abbreviations

Chol	Cholesterol
DCP	Dicetyl Phosphate
Dex-FITC 4k/150k	Fluorescein Isothiocyanate-Dextran molecular weight 4000/ 150 000
DLPC	1,2-didodecanoyl- <i>sn</i> -glycero-3- phosphocholine
DLS	Dynamic Light Scattering
DMPC	1,2-ditetradecanoyl- <i>sn</i> -glycero-3- phosphocholine
DOPC	1,2-di-(9Z-octadecenoyl)- <i>sn</i> -glycero-3- phosphocholine

DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DPPC	1,2-dihexadecanoyl- <i>sn</i> -glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
EE	Encapsulation Efficiency
EggPC	L- α -phosphatidylcholine (Egg, Chicken)
EPR	Enhanced Permeation and Retention
EtOH	Ethanol
FRR	Flow Rate Ratio
GUV	Giant Unilamellar Vesicles
HPLC	High Performance Liquid Chromatography
IPA	Isopropyl Alcohol
LNP	Lipid Nanoparticle/ Solid Lipid Nanoparticle
LUV	Large Unilamellar Vesicles
MEMS	Micro-Electro-Mechanical Systems
MLV	Multilamellar Vesicles
MVV	Multivesicular Vesicles
NLG	NanoLipoGel
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index
PEG	Polyethylene glycol
PEG-DSPE 2000	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)
POPC	2-Oleoyl-1-palmitoyl- <i>sn</i> -glycero-3-phosphocholine
RES	Reticuloendothelial system
SAXS	Small angle X-ray scattering
SHM	Staggered Herringbone mixer
SUV	Single Unilamellar Vesicles

TFR	Total Flow Rate
T _m	Transition temperature

Chapter 1 Introduction

In this chapter, the background, current challenges and motivation for this work was discussed. The problem statement and significance of this project was evaluated and explained. The hypothesis was discussed with its objectives and scope. An overview of thesis chapters was revealed and novelty of this PhD was highlighted.

1.1 Background

Since the discovery of liposomes in 1965 [1], research work has focused on using liposomes as carriers for therapeutics. In 1974, the first paper detailing liposomes as an immune adjuvant was published [2]. Conventional fabrication methods such as thin film hydration and ethanol injections are widely adopted for formation of liposomes. However, the conventional methods of liposome manufacture offer limited control on the outcomes of the product. It is also plagued by problems such as multiple post processing issues [3], lack of reproducibility, non-homogeneity of size [4] as well as low encapsulation rate of payloads, especially hydrophilic entities [5].

A plethora of reasons from compositions to fabrication methods affect the fate of the drugs within these liposomes [6]. More work can be done to improve encapsulation efficiency of the therapeutics, through exploring the points as stated above.

Conventional fabrication methods can be generally grouped into 2 categories; they are mechanical and solvent displacement. These methods utilize the ‘top down’ approach, where the particles are made non-homogeneously, followed up subsequent down-sizing process to help get the particles to the required size. Post processing problems such as loss of active ingredients through membrane filtration and lack of reproducibility hamper further development of these methods.

Other than the problems posed in conventional fabrication methods, lipid composition also plays an important role in drug/ bioactives loading. Many attempts to maximise drug loading through various lipid choice and fabrication conditions were made to improve loading outcome and reproducibility.

1.2 Significance and Problem Statement

Exploring the effects of both compositions and the fabrication methods on size, encapsulation efficiency and release profiles are the aims of this report. Composition of these nanocarriers, using lipids as a reference, will be considered in this report. For lipids, characteristics such as chain length, saturation, charge, concentration as well as the involvement of additives have profound impacts on the size, encapsulation efficiency, morphology, release profiles and clearance rates [7], [8].

Microfluidics as a platform of liposome fabrication may elevate these problems because of its precise control over liposome size, while reducing non-homogeneity. Also, it is believed to increase interaction between the drugs and lipids during formation of nanoparticles, thus increasing loading. Due to the nature of the microfluidic self-assembly, most lipids are recovered, resulting in high yield.

Although there are various studies on microfluidic chip architecture as well as process parameters study, this new approach lacks a systematic study for lipid formulations, and an understanding between this top down and bottom up approach.

The motivation of this study therefore lies in understanding how lipid compositions, external energy input and process parameters affect the fate of nanoparticles out from the microfluidic system.

1.3 Hypothesis

Conventional methods for liposome fabrication often involves size-reduction steps, and/or have reproducibility issues from different operators. Microfluidics on the other hand, eliminates these problems by virtue of design and nanoprecipitation principles. However, little studies were performed to verify if liposome formation is universal for all lipid types even though this technique requires the use of organic solvents. Here it is crucial to fill this knowledge gap, and verify if liposome formation is truly universal. It was hypothesized to be true, but with limiting conditions. This hypothesis was brought further that microfluidic approach with tuneable parameters can be optimized to yield reproducible formulations where these parameters refer to lipid compositions, operating conditions such as process and storage temperature, as well as process parameters which is the flow rate ratio and the total flow rate of the organic and aqueous solvents.

1.4 Objectives and Scope

The aim of this project is to study the effect of lipid formulation and process parameters on the physical characteristics of liposomes fabricated using microfluidics. In order to prove the hypothesis, the study will be broken into three parts:

1. A systematic analysis of lipid formulations will first be conducted using the microfluidic system.
2. Mechanistic and additives study will be conducted to understand the principles behind forming such nanoparticles using this system.

3. Drug components will be utilised to compare various lipid formulation to its encapsulation efficiency and release profile

This study will consider unsaturated and saturated lipid types with varying chain length. Process parameters such as Flow Rate Ratio and Total Flow Rate will be limited to 1.5:1 to 5:1, 2ml/min to 12ml/min. The study will only consider single drug encapsulation. Drug encapsulation and cumulative release rates will be used to assess the effectiveness of the microfluidic-directed process.

1.5 Dissertation Overview

This thesis is arranged into 7 chapters as follows:

Chapter 1 provides the research background, significance, objectives, research outcomes and novelty

Chapter 2 reviews literature concerning liposomes characteristics, conventional fabrication methods of liposomes, differences between top-down and bottom up fabrication process, general understanding of microfluidic technologies in liposome fabrication and current research progress in novel liposome fabrication

Chapter 3 discusses the materials and methods used in the studies. Selected methods were justified and described fundamentally

Chapter 4 presents the results and discussion to a bench screening of liposomes fabricated using microfluidics. The effect of lipid formulation and process parameters on size of liposomes were discussed

Chapter 5 presents the results and discussion to a fundamental understanding of interdigitation. Lipid interdigitation due to presence of organic solvent was discussed. The effects of interdigitation on the physical properties of liposomes were studied.

Chapter 6 provides the results and discussion of drug encapsulation in liposomes. Hydrophobic model drug Latanoprost was used and Dextran-Fitc was used as the hydrophilic model drug. The effect of fabrication method, lipid formulation and process parameter on drug encapsulation was discussed.

Chapter 7 relates the result of the chapter 4-6 and discusses the feasibility of using microfluidics for liposome fabrication. Future developments with regard to novel nanocarriers fabrication were discussed.

1.6 Outcomes and Novelty of Study

Novel outcomes from this research study are:

1. Knowledge of lipid formulations and operating conditions suitable for liposome fabrication using microfluidics
2. Relating the effect of interdigitation to microfluidic-fabricated liposomes and prove that interdigitation was a result of using organic solvent in microfluidics
3. Understanding the effects of fabrication methods on hydrophobic drug or hydrophilic drug loaded liposomes
4. Show that microfluidic-fabricated liposomes were superior than thin film hydrated liposomes in encapsulation of high MW drug, at 150 kDa.

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Chapter 2 Literature Review

This chapter provides a review on liposomes classifications, current fabrication techniques and drug carrying properties of a liposome. Liposome fabrication techniques will be split into two categories, bulk or top-down process and solvent displacement or bottom-up process. The discussion of this two provides the landscape of how microfluidics is superior, by encompassing the benefits of the two methods combined. In addition, basic understanding of microfluidics like flow profile and micromixer characteristics were discussed. Finally, the current progress in chip design was studied and the microchip used in this PhD study was described.

2.1 Liposomes

History, Classification and size effect of liposomes

Liposomes were first discovered in 1965 by Alec Bangham [1], but were only suggested as a drug delivery agent in 1974 by Gregory [2]. While it was a short paper, it provided critical appraisal for liposomes to be used as immunological adjuvants. Other forms of liposomal research subsequently took off, with several formulations in the market or clinical trials [3].

Classification of liposomes

A liposome consists of lipids, either occurring naturally or through synthetic methods. They are amphiphilic in nature, containing a hydrophobic tail and a hydrophilic head. Depending on the molecular shape, they can form various aggregates like micelle, bilayer or reverse micelle structures.[4]

Liposomes can be defined as broadly as 2 categories, multi-lamellar and unilamellar. In the latter, there is only 1 bilayer membrane present in the vesicle. Also, different sizes also govern the type of unilamellar structures. Figure 2.1a shows the structure of the bilayer, where the hydrophobic and hydrophilic parts align. Within unilamellar liposomes, where only a single lipid bilayer and a large aqueous core exist, there are various categories such as (Figure 2.1c):

Single Unilamellar liposomes (SUV) - 20-100nm

Large Unilamellar liposomes (LUV) - 100-250nm

Giant Unilamellar liposomes (GUV) - >1000nm

Under multi-lamellar structures:

Multi-lamellar vesicles (MLV) - 500-5 μ m,

Multi-vesicular vesicles (MVV) - >1000nm

MLVs and MVVs consist of several lipid bilayers with a smaller aqueous space. [5]

Because of the structure and amount of hydrophobic clusters, Unilamellar liposome favour encapsulation of hydrophilic drug while multilamellar liposomes favour lipophilic drugs.[6] Figure 2.1b shows the aqueous regions and the lipophilic regions which favours different types of payloads.

Figure 2.1: Liposome and the bilayer characteristics. a) Bilayer structure showing the hydrophobic and hydrophilic regions. b) Liposome structure with hydrophilic drug in the aqueous core and lipophilic drug within the bilayer. c) Various liposome size ranges. Pictures adopted from (Laouini, *et al.*, 2012) describing various liposome sizes and entrapment of lipophilic/hydrophilic drugs.

Characterization of lipids

Lipids consist of hydrophobic tails and hydrophilic headgroups, but these amphiphilic molecules exist in various forms. Table 2.1 shows the classification and examples of lipids used in various formulations. In fully saturated lipids, there are no double bonds within the hydrophobic tail. Bilayers composed of these lipids are usually rigid, and has a higher transition temperature as opposed to an unsaturated lipid of the same chain length. In an unsaturated lipid chain, there is a unsaturated carbon bond in the hydrophobic tail. There is increased mobility in the hydrophobic chains due to the presence of ‘kinks’, or the rotational movement at this unsaturated carbon atom. In liposome self-assembly techniques involving these lipids, it is easier for the lipids to bend into the spherical structure as the lowest energy conformation [7].

Apart from hydrophobic modifications, hydrophilic headgroups can be charged to improve interactions between various drugs. For example, cationic DOTAP can interact with anionic moieties such as siRNA to improve encapsulation efficiency [8], while anionic lipids can bind to cancer cells to elicit greater drug delivery response [9].

Table 2.1: Lipid physical characteristics

Lipid name	Saturation	Chain Length	Charge	Mw (g/mol)	Tm (°C)
C ₃₂ H ₆₄ NO ₈ P (DLPC)	Fully Saturated	C12:0	Neutral	621.826	-2°C
C ₃₆ H ₇₂ NO ₈ P (DMPC)	Fully Saturated	C14:0	Neutral	677.933	24°C
C ₄₀ H ₈₀ NO ₈ P (DPPC)	Fully Saturated	C16:0	Neutral	734.039	41°C
C ₄₄ H ₈₈ NO ₈ P (DSPC)	Fully Saturated	C18:0	Neutral	783.774	53°C
C ₄₂ H ₈₂ NO ₈ P (POPC)	Unsaturated carbon on one C18 chain	C18:1/C16:0	Neutral	770.123	-15°C
C ₄₄ H ₈₄ NO ₈ P (DOPC)	Unsaturated carbon on both C18 chain	C18:1/C18:1	Neutral	786.113	-17°C
C ₄₂ H ₈₀ NO ₄ Cl (DOTAP)	Unsaturated carbon on both C18 chain	C18:1/C18:1	Cationic	698	-17°C

Liposomes as drug delivery carriers

As liposomes are amphiphilic in nature, they make good carriers for drug of poor solubility. With a large amount of pharmaceutical drugs being hydrophobic in nature, use of liposomes become an interesting consideration. Low solubility drugs have low bioavailability and results

in poor absorption in the body [10], [11]. These drugs aggregate upon administration, resulting in blood vessels embolization and possible failure of the respiratory system, leading to infarction or stroke. Furthermore, the aggregation will lead to increased toxicity in the local region and poor systemic bioavailability [12]. Hence, liposome formulations are urgently required. A way to enhance solubility include pH adjustments of ionisable drugs. However most drugs are non-ionisable and non-polar [10]. Solubility enhancers or co-solvents such as polyethylene glycol 400 are used to improve sparingly soluble drugs, as studied by Seedher [13]. Another method is solid dispersions to improve oral bioavailability of low soluble drugs, but they often encounter problems of processing or storage [14]. Liposome formulations present themselves as one of the most suitable solutions, with protection from external aqueous environment and reduced degradation or aggregation at the local site. In addition, lipids can be tuned accordingly for the drug. Specifications like chain length, saturation, additives and charge can be manipulated. For example, a lipid with longer chain lengths has been shown to improve solubility due to having higher lipophilic volume. Additives such as cholesterol have been well known to stabilize liposome formulations. Cholesterol inserts itself into the interfacial area of the liposome, preventing movement and sliding of the bilayers, leading to increased stability and lesser drug loss during systemic circulation. It has been shown that presence of cholesterol changes the drug release profile from a zero-order (no cholesterol present) to a first-order (11-33 mol% of cholesterol present). However in exchange for this stability, cholesterol does reduce the capacity of bilayer to load drug encapsulation efficiency [15], [16] .

Types of drug loading methods

Passive drug loading vs active drug loading

In drug delivery, there are 2 general categories of encapsulation profile. Firstly, passive loading describes the procedure where the formation of liposomes and loading of drugs are concurrent. Hydrophilic drugs are dispersed within the rehydrating aqueous medium, while the hydrophobic drugs are usually embedded within the bilayer during the film forming process. The bilayer containing the hydrophobic drug is then rehydrated with an aqueous phase to form liposomes [17].

For active loading, liposomes are first fabricated with a transmembrane gradient. The gradient can be in the form of pH, or salt concentration. This gradient describes the difference between the aqueous core of the liposomes and the surrounding medium. For an active loading, a

prerequisite would be for the drug to be amphiphatic ie; able to exist in both charged and neutral form. An example would be catecholamine, as described by Deamer and Nicols [18] Liposomes were fabricated in a low pH environment, before being exposed to a high pH solvent with the drug dissolved in it. As the drug existed in the free form at an alkaline pH, it was able to diffuse through the bilayer easily. When it entered the aqueous core, it was quickly protonated due to the low pH of the environment. As the catecholamine now exists as an ion, it is unable to diffuse through the bilayer of the liposomes, effectively being trapped.

In passive loading, trapping efficiency is determined by many physical characteristics such as size of liposome, ratio of drugs and lipids, and procedure of forming the liposomes. Because there are usually no driving forces for the drugs to remain within the core of the liposome, low encapsulation efficiency is often expected for hydrophilic drugs. For hydrophobic drugs, an optimal drug/lipid ratio must be determined before loading can proceed. In addition, the partition coefficient of the drug, which refers to the affinity between the drug and the bilayer, plays a crucial role in the lipid formulation. Partition coefficient is affected by the bilayer and the type of drug used so the trapping efficiency for a liposome carrying hydrophobic drug is dependent on this parameter [17].

Size effects of liposomes

The fate of liposomes in vivo depends on its size. Clearance from the reticuloendothelial system (RES), which includes organs like liver, spleen, kidney, lungs, bone marrow and lymph nodes, promptly occurs when liposome size is greater than 200nm. Between 200nm to 800nm, there was no significant difference between the rates of clearance whereas no enhancement in hepatic uptake was observed for liposome sizes below 200nm [19]. The manner where the liposomes are uptake by phagocytic cells (macrophages, monocytes, neutrophils and dendritic cells) is through opsonisation, where unspecific proteins label the liposomes and increase their recognition to the phagocytic cells. They are then quickly degraded upon reaching the specific organs.

In order to escape recognition, one method is to modify the surface of the liposome using Polyethylene glycol (PEG). This increases the hydrophilicity of the particle, which has shown to have much lesser uptake by the phagocytic system. If a liposome has this 'stealth' capability then it is subjected to the Enhanced Permeation and Retention (EPR) effect [3]. To understand EPR, the propagation of an inflamed site must be understood. An inflamed site or a tumor growth requires an abnormally large amount of nutrient to propagate its growth. Newly

constructed blood vessels around this site are generally not ideal, with a leaky vasculature. It is also constructed to allow an influx of nutrients in, while preventing escape of them. If a particle containing therapeutic payloads is able to remain in circulation within the body for an extended period of time, it has a higher chance of being trapped within the leaky vasculatures. Poor network of blood vessels allow particles to get trapped within it easily and not escape. This allows particles to enter this leaky vasculature and reach its therapeutic effect. The size of these fenestrations are 300nm to 4700nm [20]. As PEG creates a water-rich shell protecting the core from being recognized by the phagocytic system, it improves the circulation time of the particle in the body. As such, PEG improves the EPR effect.

Apart from PEG stabilizers, cholesterol has been explained as liposome stabilizers adding many advantages to the system [21]. It was explained as imparting rigidity to the unsaturated lipids, so they are less permeable to stresses [22]. However, cholesterol performs another role in saturated lipids where they are at the gel state in ambient temperature. In this, cholesterol helps to disrupt the bilayers and induce packing defects [23]. These packing defects may have an impact on nanoparticle formation by inducing curvature to this originally flat bilayer.

2.2 Liposomes Fabrication Methods

2.2.1 Film Hydration

Traditional liposome production techniques are first described by Alec Bangham [1], known as the thin film hydration method. A mixture of lipids of interest are dissolved in an organic solvent, and the solvent was removed through the use of a rotary evaporator. To ensure complete removal of organic solvent, Nitrogen would be flushed through. A thin and dry lipid film would then be formed on the walls of the flask. An aqueous buffer solution would then be added under agitation at a temperature higher than the lipid transition temperature. After the film is completely hydrated into a heterogenous population of multilamellar liposomes, several size reduction techniques help to reduce it into a relatively monodispersed population. These techniques are usually sonication for SUVs or extrusion for LUVs.[5]

Sonication

Two types of equipment are usually used for sonication, the probe and bath sonicator. They are sonicated under room pressure and adjusted temperature depending on the conditions required.

Probe sonicator operates by inserting the tip into the liposome solution, and has a very high energy input. This is usually accompanied with an ice bath to prevent overheating and degradation of the final product. Even through this process of cooling, lipids can still be esterified up to 5% after an hour. Also, the probe may also shed off, leading to titanium poisoning of the solution.

Bath sonicator is a milder process compared to probe sonicator, in terms of temperature control. The liposome solution is usually put in a suitable holder and sonicated using defined temperature and time.

Although it is easy to operate and is one of the most common methods of fabrication, the disadvantages of this method are low encapsulation efficiency, degradation of phospholipids and active pharmaceutical ingredient due to heat, titanium poisoning from probe tip, and a heterogenous population of particles due to inefficiency of sonication.[24]

Extrusion/French Press

Another commonly used method to reduce size is through the French pressure cell or extrusion. After rehydration, the liposome dispersion is extruded through an orifice. They are usually extruded sequentially from a largest pore size membrane, typically from 1 to 0.2 μ m. Also, it was important to ensure that there was a good seal, else leakage of samples will result. Non encapsulated materials would then be removed through dialysis. [25]

This method results in monodispersed particles [6], but it is difficult to maintain high temperatures, and because of its low volume (<50ml), it has batch to batch variability, leading to low reproducibility. Among them, loss of drugs during extrusion compound the disadvantages this system has [24].

Top down approaches involve producing the particles from an initial larger size, and subsequently reducing its size. It usually involves an external energy source for the process. A typical traditional method is thin film hydration followed by sonication or extrusion. Bottom up approaches refer to producing the particles from lipid molecules, and self-assemble in various conditions to get the final size. [44]

Industrial methods for liposome preparations

While extensive research on liposomes have been carried out, few have been able to extend towards clinical use, either from clinical trials failure or inability for scale up. The instability in terms of reliability and reproducibility of the liposome formulation hampers the progress of liposomal therapeutics. Also, lack of equipment and expertise also cause production to be unreliable. Only after addressing the first two issues, then clinical trials can proceed. Within this clinical trials, suitable control groups have to be setup to ensure comparable results of preclinical to clinical, and by then the cost of reaching this stage might be a limitation.[3] Table 2.2 shows the number of clinical liposomal formulations emerging from conventional fabrication methods. One of the pioneers, Doxil, is still currently explored as therapeutics for other forms of disease. However over the past 5 decades only 15 formulations were successful in terms of drug encapsulation, pharmacokinetics and showed industrial scaling potentials [3]. The lack of formulations highlights the urgency of making liposomes in industrial scales.

Heating

This modified heating method utilizes purely heat and stirring to produce liposomes of industrial scales, without using any organic solvent. In this method, the lipids (DPPC: DCP: Chol 70:20:10) were first hydrated for 1-2 hours in aqueous buffers separately, mixed at high temperatures (120 °C for cholesterol, 60°C for DPPC, DCP) in the presence of glycerol and brought down to room temperature. Glycerol enhances liposomal stability by preventing coagulation and sedimentation. Also, it is biocompatible, water soluble and non-toxic to the body, so additional removal process is not necessary. Pharmaceutical actives can be added in the initial step before mixing, during mixing or after it has cooled down. Mozafari *et al* reported high plasmid DNA entrapment efficiency (81%) using this method. However, reported sizes were large (>500nm) and extrusion was likely required to process them into smaller vesicles.[26], [27]

Serial	Drug	Drug and lipid formulation	Disease	Company	Year
1	Epaxal®	Inactivated hepatitis A virus (strain RGSB) DOPC:DOPE (75:25 molar ratio)	Hepatitis A	Crucell, Berna Biotech	1993
2	Doxil®	Doxorubicin HSPC:Cholesterol:PEG 2000-DSPE (56:39:5 molar ratio)	Ovarian, breast cancer, Kaposi's sarcoma	Sequus Pharmaceuticals	1995
3	Abelcet®	Amphotericin B DMPC:DMPG (7:3 molar ratio)	Invasive severe fungal infections	Sigma-Tau Pharmaceuticals	1995
4	DaunoXome®	Daunorubicin DSPC and Cholesterol (2:1 molar ratio)	AIDS-related Kaposi's sarcoma	NeXstar Pharmaceuticals	1996
5	Amphotec®	Amphotericin B Cholesteryl sulphate:Amphotericin B (1:1 molar ratio)	Severe fungal infections	Ben Venue Laboratories Inc.	1996
6	Ambisome®	Amphotericin B HSPC:DSPG:Cholesterol:Amphotericin B (2:0.8:1:0.4 molar ratio)	Presumed fungal infections	Astellas Pharma	1997
7	Inflexal® V	Inactivated hemagglutinine of Influenza virus strains A and B DOPC:DOPE (75:25 molar ratio)	Influenza	Crucell, Berna Biotech	1997
8	Depocyt®	Spinal Cytarabine/Ara-C DOPC, DPPG, Cholesterol and Triolein	Neoplastic meningitis	SkyPharma Inc.	1999
9	Myocet®	Doxorubicin EPC:Cholesterol (55:45 molar ratio)	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan Pharmaceuticals	2000
10	Visudyne®	Verteporphin Verteporphin:DMPC and EPG (1:8 molar ratio)	Choroidal neovascularisation	Novartis	2000
11	Mepact®	Mifamurtide DOPS:POPC (3:7 molar ratio)	High-grade, resectable, non- metastatic osteosarcoma	Takeda Pharmaceutical Limited	2004

12	DepoDur™	Morphine sulfate DOPC, DPPG, Cholesterol and Triolein	Pain management	SkyPharma Inc.	2004
13	Exparel®	Bupivacaine DEPC, DPPG, Cholesterol and Tricaprylin	Pain management	Pacira Pharmaceuticals, Inc.	2011
14	Marqibo®	Vincristine SM:Cholesterol (60:40 molar ratio)	Acute lymphoblastic leukaemia	Talon Therapeutics, Inc.	2012
15	Onivyde™	Irinotecan DSPC:MPEG-2000:DSPE (3:2:0.015 molar ratio)	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceuticals Inc.	2015

Table 2.2: Commercially available liposome formulations [8]

Spray drying

Spray-drying method is another method suited for large-scale production of liposomes owing to its rapid and single-step fashion. Like most starting processes, lipids were first dissolved in chloroform. The lipids were then sprayed into a drying chamber using a spray nozzle with a specified orifice size. Hot air was also accompanied into the drying chamber. Although the spray-dried samples should be collected on the reservoir below the chamber, some samples adhered to the walls, and this phenomenon was dependent on the types of lipid. As such, Mannitol was added into the organic solvent as it helps the spray-dried lipids rehydrate and prevents them from adhering to the wall. Hiroshi *et al* proposed this method and also discussed factors affecting the lipid mixture recovery. It was also suggested that the lipid composition affected lipid recovery as the transition temperature of the lipids affected how they would have strong adhesion to the chamber walls. This was observed even in the presence of mannitol, for the lipid EggPC [28].

In another study proposed by Skalko-Basnet *et al*, an improvement to the stability of these liposomes were made through incorporating cyclodextrins. Cyclodextrins act as a stabilizer in combination with mannitol, as well as being a drug carrier. It forms a complex with metronidazole, a poorly soluble drug in both hydrophilic and lipophilic solvent. After spray drying, samples were rehydrated with aqueous buffers and further agitation produced liposomes with relatively large vesicle sizes between 300-500nm. It was shown in that there were no significant differences observed in size between freshly prepared and older (>1 year) spray-dried samples. This signifies the stability of these particles. In addition, the encapsulation efficiency reported was relatively high at 47%. However, further improvements can be expected in terms of the size of the liposomes as well as recovery of lipids in the spray-drying chamber [29].

Freeze-drying

This method involves freeze-drying a monophasic solution containing the liposomes. The liposomes prepared via a double emulsion method. Figure 2.2.1 shows the schematics of the double emulsion formation. Mixture of cyclohexane, dichloromethane and diethyl ether (4:1:1, v/v/v) were used to dissolve lipids and PEG (O). Sucrose spiked aqueous buffer (W1) was used. Sucrose acts as a cryoprotectant to prevent rupturing of the membranes during the freezing process. Probe sonication was performed at 200 w for 3 mins until a clear mixture, or a uniformly opalescent microemulsion was formed. More aqueous buffer (W2) was added and

emulsified at 18000 rpm for 30 s for the double emulsion to form. After filtration, the mixture was frozen at -80 °C for 4h, dried at -50 °C for 4 h, then -37 °C and -20 °C for 2 hours each, followed by a final drying at 20 °C for 4 h. When required, the samples were rehydrated with an aqueous buffer above its transition temperature. Unfortunately, low encapsulation among the complexity of the operation plagues this promising technique [30].

Figure 2.2.1: Schematic diagram for the formation of Freeze-drying double emulsion liposomes. a) The organic phase containing lipids and the aqueous phase containing lyoprotectants. b) The formation of first W/O emulsions. c) The formation of W/O/W double emulsions d) The formation of solid spheres after freeze-drying, protected by sugar molecules. e-f) The formation of liposomes upon rehydration with water. “-“ and “+” represents water and oil molecules [30].

Electroformation

Electroformation involves the use of an electric field for the fabrication of liposomes. Dry lipid films are deposited on a conductive substrate, 2 electrodes made of indium tin oxide (ITO). An aqueous solution containing sucrose, was added as the rehydration buffer. As an alternating

electric field is applied on these lipid films, swelling occurs and the bilayer is rehydrated. This swelling process is similar to the conventional hydration method but produces Giant Unilamellar vesicles (GUVs). It involves very little technical expertise, generates unilamellar vesicles that are relatively monodisperse. However, the cost of the electrodes limit the industrial scalability of this method [31], [32]. To counteract this limitation, Pereno *et al.* suggested the use of stainless steel but is currently used for biophysical investigations of the membrane instead [33].

Supercritical fluids Technology

Supercritical fluids, like the name implies, uses a solvent at its supercritical state. This retains the dissolubility for a liquid and the mass transfer properties for a gas. A popular choice for the supercritical fluid is carbon dioxide, CO₂. The wide application is due to its low critical temperature of 304 K and a moderate pressure of 7.4 MPa. It is also attractive due to its favourable physical properties. It is non-flammable, non-toxic, miscible with most organic solvent and recovered easily post processing. The recovery is made easy due to its small and linear structure, which allows diffuses out faster than liquid solvents.

An example of supercritical fluid processing consists of having 2 streams, 1 containing the lipid dissolved in small amounts of organic solvent and the supercritical fluid, and the other aqueous buffer. After cycling the lipid stream to ensure homogeneity, it is expanded to a low-pressure state in the presence of aqueous buffer. This precipitates the lipid into the aqueous buffer, allowing formation of liposomes in the process. It has been shown that this process produces nano to micron sized particles [34], [35].

2.2.2 Solvent Displacement

Ethanol Injection

Injection of organic solvent consisting of lipids into an aqueous solvent, triggering self-assembly into particles is known as ethanol injection. It was first described by Batzri and Korn [36] in 1973 to produce SUVs without sonication. Several advantages are relative homogeneity and ease of operation. However one major drawback is the removal of ethanol after operations. While it is not required for certain applications to remove ethanol, dialysis may not require ethanol completely. Another drawback is the requirement of an organic solvent miscible in aqueous buffer, hence ethanol is the most common solvent used. This leads to a limited choice for lipids to be dissolved in, as some of them are sparingly soluble in such solvents [6].

Modified ethanol injection method

The modified ethanol injection method is an improvement to conventional injection method, with attempts to overcome the shortcomings. An automated system proposed by Justo and Moraes serves to improve the scalability of this method from the lab scale to the industrial scale. Instead of using a syringe, an automated syringe pump was proposed, while keeping the ethanol content below 5%. Jeffs *et al* [37] approached ethanol injection in another way, by increasing the ethanoic content to 45% and removing afterwards, citing higher encapsulation of DNA. In his modified ethanol injection method, both lipids dissolved in ethanol and aqueous buffer solutions were both heated and mixed using a peristaltic pump. The resultant ethanol concentration increases to 45% and the liposomal solution may be unstable. As such, the vesicles formed are immediately stabilized by diluting the liposomes with the same buffer solution. After the dilution, the unencapsulated DNA was removed via charged membrane filtration while the organic solvent was removed using a tangential flow ultrafiltration. With this method, they reported an encapsulation of between 80 to 90% of DNA [38].

Reverse Phase Evaporation

Reverse phase evaporation combines both the principles of thin film hydration with solvent displacement effects. After casting the thin film on the walls of the flask, the lipids are re-dissolved in an organic solvent, where the reversed phase vesicles are formed. The aqueous phase will then be added, to form a two-phased system. It is then sonicated at a low temperature for a short period of time until only one phase can be observed. The system then undergoes

another round of rotary evaporation to remove the organic phase, leaving behind the vesicles in the aqueous buffer. Although it has managed to maintain homogeneity in size as well as increased encapsulation efficiency, the laborious process and the potential use of harsh organic solvents would hamper the extensive use of this method. Also, the use of such organic solvents may denature proteins as an added disadvantage [39].

2.3 Microfluidics

Microfluidics technology is based on precise movement of fluids within a constrained volume or channel to ensure controlled mixing. The term ‘lab on a chip’ refers to its ability to encompass the capabilities of a laboratory into a chip. The inception came about through the combination of electronic circuits, mechanical force and fluid flow, in a miniature device known as micro-electro-mechanical systems (MEMS) [44]. Ever since, it has been used for biological [40], chemistry[41] , diagnostics [42] and are gaining traction in drug delivery systems.

Principles

Methods that attempt to form liposomes in the bulk phases usually result in a polydispersed population, where there is little control over the formation condition [43]. There is precise control over various parameters in the microfluidic setup, which enables size and homogeneity of the liposomes to be scrutinized. The main mechanism for this microfluidic process to form liposome is through nanoprecipitation, a well-known technique that involves a ‘bottom up’ approach. This process was introduced by Fessi *et al* in 1989 for polymers [44]. In essence, it involves two miscible phase; an aqueous buffer phase and an organic solvent phase. Lipids are solvated in the organic phase, and go through nucleation and growth as the aqueous buffer thins the organic solvent. Supersaturation is the driving force towards the formation of nanoparticles, when the solubility of the particle makes it unable to remain sufficiently solvated. Growth of this particles take place until it reaches colloidal stability, a property dependent on stirring, molecular weight, solvent pairings among many others [45].

Also known as the solvent displacement method owing to its systemic manner of precipitating the particle into the non-solvent, this method is easy, fast, cheap and do not require the use of excessive toxic organic solvents [46].

2.3.1 Dimensionless Numbers

The ability to achieve the desired nanoparticle depends mostly on the fluid flow profile. They are governed by a series of dimensionless numbers, relating various characteristics of the fluid

as well as the channel geometry. A few terms that relate them are Reynolds Number (Re), which relates density, velocity and characteristic length to viscosity, as well as Peclet number (Pe), which relates diffusion and advection domination.

Reynolds number:

Equation 2.3.a: Reynolds Number

Reynolds number

$$Re = \frac{\rho u l}{\mu} = \frac{\rho u D_h}{\mu} = \frac{u D_h}{\nu}$$

where

ρ = Density of fluid

u = Velocity of fluid

l = Characteristic length of pipe

D_h = Hydraulic Channel Diameter = $4A/P$, where A = cross sectional area for non-cylindrical pipes, P is the wetted perimeter

μ = Dynamic viscosity of fluid

ν = kinematic viscosity of fluid (μ/ρ)

Equation 2.3a shows the calculation of Reynolds number to understand flow characteristics. Figure 2.3.1 depicts the different flow profiles as the Reynold number increases. To understand this set of dimensionless number, it should be useful to note several parameters:

For Reynolds number lesser than 1000, the flow is laminar, and there are no mixing along the axial directions. For Reynolds number greater than 10^3 , there is a generation of vortexes that cause the fluid flow to be uncontrolled, these vortex trails or eddies are called turbulences.

In the case of microfluidics operation, owing to the small characteristic length, the Reynolds number is kept below 100, where such eddies are not pronounced [47].

Figure 2.3.1: Reynolds number description. Laminar or creeping flow for Reynolds number below 1000. Generation of eddies and vortexes for Reynolds number above 4000. Reynolds number between 1000 and 4000 represent a mixture of both.

Peclet number

$$Pe = \frac{ul}{D} \quad \text{And} \quad Pe \, l = \frac{ul}{D} \times l$$

Where

And

u = Velocity of fluid

l = Characteristic width of channel,

D = Diffusion coefficient

Equation 2.3.b: Peclet number calculation. Distance required for particles to mix. Pe L represents the distance for particles to travel for full mixing to occur.

Equation 2.3b describes the ratio between diffusion and advection. Usually this ratio is large in microfluidic processes owing to the small diffusion coefficient with respect to the velocity and channel width ($Pe > 100$). This also means that the mixing should be advection dominated, wherein bulk movement of the fluid to induce mixing should overcome random diffusion of the species. In other words, if the Pe is small, diffusion occurs quickly; if Pe is large, convection induces mixing [47].

2.3.2 Microfluidic Devices

2.3.3 Micromixer Flow Characteristics

From the previous section, calculation of the Reynolds number defines the flow stream to be laminar. There should be no crossing of each velocity lines. Since the fluid flow is parallel, mass transfer can only be diffusion dominated.

With the Reynolds number asserting diffusion domination while Peclet number suggesting convection domination, it may seem contradictory to have a system where both mass transfer mechanism are dominant. This is a classic case of a Low-Reynolds-Number, High-Peclet-Number Limit. If Pe was small or if there was greater interaction of the flow fields, diffusion

time can be shortened. If Pe is high, and little can be done to alter the flow field, then mixing can take a very long time or over a very long distance.

Consider a simple example:

A 100- μm wide channel with a particle velocity of 100 $\mu\text{m/s}$ has a diffusion coefficient of $D = 1 \times 10^{-16} \text{ m}^2/\text{s}$ (typical of 1 μm particle). The Peclet number is 10^8 , and the length required for mixing to occur in this channel would be 10^{10} micron, or 10km.

To counteract this problem, methods were devised to reduce the flow path to increase diffusion. Examples are passive and active micromixers, which provide a greater driving force or a reduction in flow path[48].

2.4 Microchip Architecture

2.4.1 Mixer-assisted Microfluidics: Active Micromixer

To improve mixing efficiency while preventing excessively long diffusion paths, several methods are employed. There are two classifications of such mixers, namely active and passive mixers. Active mixers rely on external energy to generate perturbations and improve mixing, while passive mixers rely heavily on geometry of the channels or flow field to decrease diffusion length.

Examples of external energy input are ultrasound, acoustic, vibrations, electokinetic instabilities, periodic variation of flow rate, electrowetting-induced merging of droplets and mechanical manipulation in the form of impellers as well as pumps [49].

While active mixers have a higher mixing efficiency, it comes at a cost of complex engineering design as well as ease of implementation. To ensure the power source is working as designated requires a long time and intricacy in design leads to a high cost. Also, the energy from ultrasonic devices as well as high temperature could damage biological samples [50].

Passive mixers work on manipulating flow energy, such as restructuring flow to have faster mixing. Several strategies such as T and Y-shaped micromixers, Split-and-recombine or sequential lamination, structured packing flow configurations, chaotic flow or advection configuration, recirculation flow configurations or zig-zag channels, colliding jet configurations and moving droplets configuration [49], [50]. In this study, a particular geometry of Staggered Herringbone Mixer was considered due to its efficiency, ease of fabrication and operation [47].

Passive micromixer – Staggered Herringbone Mixer (SHM)

Inception of microfluidics has led research direction towards characterizing flow in miniature device, with emphasis on mixing characteristics. As discussed previously, there is difficulty in mixing solutions in a microchannel. Typical flow conditions dictate a laminar flow condition but requires fluctuation in flow pattern to enhance mixing. One example of a passive mixer is the SHM, where imperfections in the channel structure tune fluid flow to fold and stretch together, resulting in a homogenous solution within a short time span. It is a mixer based on

patterned grooves on the channel floor. This flow profile is known as the chaotic flow, or known generally as chaotic advection [47].

A schematic of SHM showing the three-dimensional twisting flow, induced helical flow due to ridges on the channel floor is shown in Figure 2.4.1. The coordinate systems represent the axes of the channel (x, y, z) and the ridges (x', y', z). The angle θ represents the orientation of the ridges with respect to the channel. The height of the ridges is represented by α , but is insignificant as compared to the average channel height h . Channel width is w and the principal wavevector is q . Angular displacement of the fluid flow is described by $\Delta\Phi$.

Figure 2.4.1: Schematic diagram of a Staggered Herringbone Mixer (SHM) [47]

Figure 2.4.2 describes the flow profile of A, where there are no ridges on the chip. Mixing was not accomplished at the end of the channel. The flow profile of B, where there are only straight ridges, showed some forms of mixing but not complete. For the flow profile of C, with the herringbone mixer design, complete mixing was observed after cycle 15, through the homogenous fluorescence of streams.

Figure 2.4.2: Performance of SHM as compared to a channel with no ridges (A), with straight ridges (B) and with the staggered herringbone structure (C) [47].

Microfluidic hydrodynamic flow focusing

Jahn *et al* first introduced the concept of hydrodynamic flow focusing technique in microfluidics in 2004, for the rapid production of nano-sized liposomes. Basically, a stream of lipid dissolved in organic solvent is sheared in the axial direction by aqueous buffers. The aqueous buffer shears the lipid stream into a thin sheet, and liposomes produced through this method can be fine-tuned by adjusting the flow ratio of organic to aqueous. In this case the organic solvent has to be miscible with the aqueous buffer so IPA with PBS was used. Figure 2.4.3 shows the inlet of focus channel. Due to the laminar flow profile, mixing was strictly at the interface of lipid and buffer. As such vesicle formation was well controlled, ensuring monodisperse sizes. Sizes reported were between 100-300 nm. Most importantly, they can be facilely controlled by altering the flow ratio between the buffer and the lipid stream. Also, no

cross mixing in either streams were observed according to the fluorescence tests performed by Jahn [51]. A further optimization was performed by changing the number of aqueous stream inlets, from 3 to 5. The additional 2 inlet serves as inlet for drugs or bioactives that are soluble in aqueous medium. Figure 2.4.3b-c shows the schematic of the 5 inlet microfluidic channel. The benefit of this design helps reduce drug losses by confining the water soluble drugs to the immediate vicinity of the organic solvent stream, where liposomes are expected to form [52].

Figure 2.4.3: Various microfluidic flow focussing technique a) 3 channel chip architecture, 2 aqueous, 1 lipid channel. b-c) Multiple aqueous channel 3-5, 1 lipid channel [52].

Vertical flow focusing

Vertical flow focusing operates on similar principles to its predecessor, hydrodynamic flow focusing, but produces a higher yield. Figure 2.4.4 shows the architecture for the VFF chip. The reason is due to its vertical stack of wide but shallow channels. Hood and Devoe have shown that producing monodisperse liposomes of 80 – 200 nm was possible using this method. In addition, they have shown high yields of 100 nm liposomes at 95 mg/h. As compared to MHF in their study, where MHF only managed less than 10 mg/h [53]. The VFF can also be automated, increasing its desirability for industrial uses. However, it is still a lab-based technique, and require fine tuning of chip interface.

Figure 2.4.4: Vertical flow focussing technique [53]

Droplet based microfluidics

Droplet based microfluidics stems from the use of a similar hydrodynamic flow focusing technique, to produce GUVs of liposomes. In comparison to the conventional hydrodynamic focus, the outlet of the device is pinched for discrete droplets to be formed. Most of these techniques employ the use of an immiscible fluids such as octanol. Emulsions are formed initially with these immiscible fluids, and they serve as template for producing liposomes [54].

2.5 Summary

Liposomes fabrication techniques have evolved over the decades since its inception by Bangham in the late 90s, to Reversed-phase evaporation techniques. As for solvent displacement techniques, simple ethanol injection methods developed by Korn has advanced towards microfluidic techniques with various architectures. Table 2.5 provides a summary of the discussed fabrication techniques. In liposomes fabrication, there are many aspects to evaluate. Various research efforts to improve the encapsulation efficiency, scalability, biocompatibility have been valiant and more work can be done to integrate the benefits of the fabrication method into a truly effective one.

Table 2.5: Comparison of various microfluidic techniques

Number	Method	Types of lipid	Solvent	Size of liposomes	EE	Scalability	Ref
1	Thin film hydration + Extrusion/Sonication	DPPC:Chol	PBS	LUVs-SUVs	Low	N	[1]
2	Thin film hydration + Heating	DPPC:DPC:Chol (70:20:10)	PBS:Gly (97:3)	MLVs	High	Y	[26-27]
3	Spray-drying	Lecithin	Mannitol/Chloroform	LUVs (300-500nm)	Low	Y	[29]
4	Freeze-drying	SPC, SPS:SPC (10:90), SPS:SPC:Chol (10:40:50)	Cyclohexane	LUVs (300-500nm)	High	N	[30]
5	Electroformation	EPC	Chloroform:MeOH (2:1)	GUVs	Low	N	[31-32]
6	Modified ethanol injection	EPC	PBS/IPA/EtOH	SUV-LUVs	Low	Y	[34-35]
7	Reverse-phase evaporation	EPC/DPPC	PBS	LUVs	High	N	[39]
8	Microfluidic hydrodynamic focusing	DMPC:Chol:DCP (50:40:10)	IPA	SUVs,LUVs (<200nm)	Low	N	[51]
9	Vertical flow focusing	DMPC:Chol:DCP (50:40:10)	EtOH	SUVs,LUVs (<200nm)	Low	N	[53]
10	Mixer assisted microfluidics (passive/active)	EPC	EtOH	SUVs,LUVs (<200nm)	High	Y	[47]

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Chapter 3 Experimental Methodology

This chapter describes the methods and materials used in this thesis. The NanoAssembler setup as well as liposome fabrication method using the equipment were described. Various characterization methods were also discussed, where physical properties such as size, zeta potential of the liposomes were measured. For drug encapsulation efficiency, the principles were discussed together with the reasons for the chosen method. Similarly, methods for measuring size, polydispersity and advanced laser microscopy were discussed and reviewed.

3.1 Materials

The lipids egg phosphatidylcholine (EPC), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC), 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were all purchased from Avanti Polar Lipids Inc. US. Cholesterol (CHOL) was purchased from Sigma Aldrich Company Ltd., Singapore. For solvent removal, a cellulose ester membrane (100kD) was used. Dex-FITC 4000, 150000 were purchased from Sigma Aldrich Company Ltd., Singapore. Latanoprost (99.5%) was purchased from Chemical testing and Calibration laboratory., Singapore.

3.2 Methodology

NanoAssemblr Setup

NanoAssemblr was obtained from Precision Nanosystems (Figure 3.2). Microfluidic chip responsible for the mixer was a staggered herringbone micromixer (SHM). Disposable syringes ranging from 1-10ml was fitted to both inlet and collected by 15ml centrifuge tubes. A heating block was attached for any intermittent heating.

Figure 3.2: NanoAssemblr Setup and microfluidic chip structure

3.3 Fabrication of liposomes by microfluidics

The fabrication of liposomes by microfluidics was conducted on the NanoAssembler Benchtop system from Precision Nanosystems (Vancouver, Canada). Microfluidic chip responsible for the mixer was a staggered herringbone micromixer (SHM) with cyclic olefins polymer channels. Selected lipids were dissolved in organic phase (methanol or ethanol) at 18 mM and injected into the right inlet of the microfluidic chip, whilst the aqueous phase (PBS; pH 7.4) is injected into the left inlet of the microfluidic chip. For mixtures of lipid, it was ensured that the initial concentration of DPPC was 18 mM. A heating block was attached for any intermittent heating. In the software, process parameters like Total Flow Rate (TFR), Flow Rate Ratio (FRR) and Total Volume can be adjusted. All FRR and TFR used were 3:1 and 12 ml/min unless otherwise specified. The resulting aqueous dispersions of liposomes formulations were collected from the outlet stream and subsequently dialyzed overnight using cellulose ester membranes against aqueous 1X PBS under 1000x dilution to remove any residual solvent. Samples were subsequently used for DSC and SAXS experiments.

3.4 Fabrication of liposomes by thin film hydration

Lipids were weighed and dissolved in 3ml of chloroform: methanol (2:1, v/v%). Organic solvents were removed through rotary evaporator under vacuum conditions of 20 mBar for 1 hour. Hydration of the lipid film was achieved by the addition of PBS or PBS/EtOH at temperature above the appropriate lipid transition temperature. Subsequently the liposomal formulation was put through the extruder for size reduction.

Extrusion procedure was prepared accordance to Olson *et al* [1]. Extruder was setup with a bath temperature of at least 20 °C above lipid transition temperature. Formulation was put through a membrane pore size of 0.2 μm , 0.1 μm , 0.05 μm for 10 times each. Samples were dialyzed overnight and used for subsequent DSC and SAXS experiments.

3.5 Liposomes Purification

Liposomes were dialysed using cellulose ester membranes against aqueous 1X PBS under 1000x buffer volume to remove solvent as well as unencapsulated drug. Dialysis were 4 hours to remove free drug. Dialysis tubing, beakers as well as clips were washed and soaked to ensure completely removal of contaminants. Liposomes produced in Chapter 6 was purified by ultracentrifugation, at 110k rpm for 1 hour at 4°C. Supernatant were removed and an equivalent amount of pure PBS was added and resuspended by pipetting.

3.6 Physical characterization

3.6.1 Dynamic Light Scattering (DLS)

Liposome size was characterized using the Malvern Zetasizer. Dynamic light scattering (DLS) was used to analyze the intensity mean diameter (z-average) and polydispersity index (PDI) of the liposomal formulations using Malvern Zetasizer Nano-ZS, (Malvern Instruments, UK). All measurements were conducted at 25°C with samples diluted 50 times with PBS. Unless specified otherwise, Liposome formulations are diluted 50 times with 1x Phosphate buffer saline at 25°C. Z-average were calculated using intensity based distribution.

For the measurement of particle size over a range of temperature, samples were equilibrated for 120 seconds prior to each particle size measurement at the specified temperature. The range of temperature measured is from 25 to 60°C with every 5°C increments.

3.6.2 Zeta Potential

Characterisation of liposome zeta potential was performed using the Malvern Zetasizer. Unless specified otherwise, Liposome formulations are diluted 50 times with 1x Phosphate buffer saline at 25°C. Total number of measurements were decided by machine and three runs were performed.

3.7 Nano Differential Scanning Calorimetry

Thermal scans were carried out using Nano DSC (TA Instruments, UK). All samples were scanned from 20 to 50 °C using a scanning rate of 1 °C/min. A capillary containing identical solvent to the sample were used for the baseline and compared with another capillary containing the sample. The temperature range of 20-60 °C was used for this study.

3.8 Small Angle X-ray Scattering

Small angle X-ray scattering patterns were obtained from a Nano-inXider (Xenocs, France) with Cu-Ka microsource (40 mm) at 30W. Samples were injected into a low-noise flow cell unit. Distance of detector to sample was 938 mm, with a 0° angle tilt. Scattering vector 2Θ was from 4.9° to 60°. The measurement was conducted at 25 °C.

3.9 Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM images of liposomes were acquired under cryogenic conditions in order to avoid drying artifacts. The samples were prepared for cryo-TEM imaging via the plunge freezing method. A 3µl drop of sample (lipid concentration of 0.9 mM) was placed onto a 300 mesh Lacey carbon grid (Ted Pella Inc., Redding, CA), blotted and then plunged into liquid ethane in order to vitrify nanolipogel particles in a thin layer of ice. The samples thus prepared were stored in liquid nitrogen prior to image acquisition to maintain vitrification. Imaging was done using Carl Zeiss Libra 120 Plus system (Carl Zeiss, Jena, Germany) at 120kV with Gatan 626 cryo-holder (Gatan, Inc., Pleasanton, CA). A 2k x 2k CCD camera (Troendle (TRS), Sharpeye) was used to record the images. Sample temperature was maintained below -170 °C throughout the experiment.

3.10 Drug Quantification

Latanoprost

Reverse phase HPLC was performed to quantify drug encapsulation and release profiles. A C18 Eclipse-XDB C18 column (5 µm, 4.6 mm ID × 250 mm) with a wavelength set according to the

required value in table 3.1. Column temperature was controlled at 30°C. All runs were isocratic. UV wavelength, injection volume, analysis time and mobile phase were varied and described in Table 3.1. For each drug quantification, a standard curve would be produced.

Table 3.1: HPLC conditions for the analysis of Latanoprost

Drug	Wavelength (nm)	Injection volume (μl)	Analysis time (min)	Mobile phase	Flow rates (ml/min)
Latanoprost	210	50	10	70:30 ACN/H ₂ O v/v	1

Encapsulation efficiency from microfluidics process as well as any post treatment process were calculated using equation 3.10a.

$$Drug\ left\ (\%) = \frac{Drug\ after\ process(mg)}{Drug\ before\ process(mg)} \times 100$$

Equation 3.10.a: Calculation for Encapsulation Efficiency of Latanoprost

Dex-FITC 4k, Dex-FITC 150k

Quantification of Dex-FITC was performed using a Tecan Infinite M200 plate reader. Samples were incubated in 80% ethanol (EtOH) and shaken vigorously for 10 minutes to rupture the lipid bilayer. 10x dilution was performed on the sample prior to measuring sample fluorescence. Excitation wavelength and absorbance wavelength was determined to be 480nm and 520nm respectively. For each drug quantification, a calibration curve was performed.

3.11 Statistical Analysis

All experiments were performed in triplicates with calculation of means and standard deviations. Statistical significance was determined by ANOVA and determined to 0.05 and 0.01 confidence intervals ($p < 0.05$) ($p < 0.01$). Tukey's range test was performed to determine statistically different groups if p value from ANOVA analysis was significant.

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Chapter 4 Fundamental understanding of liposome fabrication using microfluidics

In this chapter, an overview of how various lipid composition and process parameters affect liposome fabrication in microfluidics was given. For lipid composition, the effect of chain length, chain saturation, cholesterol, PEG-DSPE2000 on the size of the liposome was studied. The aim was to examine liposome fabrication using microfluidics, to test the initial hypothesis that microfluidics provides a universal method for all type of lipids. For the effect of additives, the ratio required for a successful liposome formulation was determined. The effects of microfluidics as a fabrication technique were discussed in this chapter.

Effect of lipid composition and process parameters on particle characteristics

4.1 Effect of lipid composition on size- Chain length

To investigate the role of lipid composition for the formation of liposomes by microfluidics, different lipid compositions were considered (Table 4.1). Liposomes were produced at a 3:1 FRR and a 12 mL/min TFR with operating temperature at 60°C. The liposome size and PDI was investigated prior solvent removal. Result in Figure 4.1 showed that liposome formation only occurred with DMPC lipid (~33 nm in diameter) with longer phospholipid alkyl chains unable to form stable liposomes. Although apparently nano liposomes were formed with longer chain length PCs (C16 and C18) as a result of microfluidic mixing at temperature above the gel-to-liquid crystalline phase transition, the initially optically-clear formulations of DPPC and DSPC turned turbid within minutes after formation, indicating temperature-triggered lipid aggregation.

Table 4.1: Lipid composition for the production of liposomes using microfluidics.

Lipids	Carbon chain length	T _m (°C)	Saturation
EPC	16 carbons	-15	16:0, 18:1
DOPC	18 carbons	-17	18:1, 18:1
DMPC	14 carbons	24	14:0
DPPC	16 carbons	41	16:0
DSPC	18 carbons	53	18:0

Figure 4.1 Effect of chain length on size (* = 0.05 CI, ** = 0.01 CI)

4.2 Effect of lipid composition on size- Chain saturation

Unsaturated lipids have similar phospholipid structure as saturated ones but contain one or two double bonds within the alkyl chain. This results in a cis-conformations of the alkyl chains, leading to 'kinks' in the otherwise straight trans- conformation [1] . With liposome formation, it has been understood that curving of the bilayer is an equilibrium between bending energy and surface energy [2]. The presence of 'kinks' increases the packing defects and lowers the bending energy required for successful liposome formulation, in contrast to saturated lipids. This also results in most unsaturated lipids having transition temperatures lower than room temperature compared to saturated lipids. In Figure 4.2, results show that nanoliposomes were able to form stable liposomes, with both EPC and DOPC having sizes below 100 nm.

Figure 4.2: Effect of chain saturation on size (* = 0.05 CI)

4.3 Effect of additives on liposome size-

4.3.1 Cholesterol

The effects of cholesterol stabilization in liposomal formulations are reducing its susceptibility towards any aggregation and decreasing fluidity of the system [3]. However, when added to a fully saturated lipid, it can impart fluidity. By inserting itself to the leaflets of the saturated alkyl chains, it improves the bilayer tendency to curve. Studies have thus shown that incorporation of cholesterol has decreased rigidity of bilayers below their transition temperature [4], and may have effects on its formation. Figure 4.3.1 shows the effect of cholesterol addition to size. Although the data suggests that sizes decreased when DPPC : cholesterol of 75:25 was used, PDI was high. Figure 4.3.1b shows the peak locations for the lipid formulations. There were presence of multiple peaks for DPPC: Cholesterol 75:25, with sizes up to 1 micron. It is only at equimolar ratio of cholesterol and DPPC where there is a reduction in size and PDI. A similar study conducted by Pan *et al.* showed that the bending modulus (K_c) of DMPC liposomes were increased four-fold when cholesterol (30 mol%) was added. This study suggests that cholesterol helps to improve liposome formation. [5] Under this condition, it is monodispersed as well.

Figure 4.3.1 a) Z-average measurement of DPPC and cholesterol in various ratios, b) size distribution by intensity of liposomes fabricated from various ratio of DPPC: Cholesterol

4.3.2 PEG-DSPE2000

Functionalization of end terminals with Polyethylene Glycol or ‘PEGylating’ is a common method to protect the carrier before it reaches the site of interest. Advantages include reducing renal clearance, decreased opsonisation by MPS, as well as increasing solubility due to PEG’s hydrophilicity [7]. In this study, it allows the system to form nanoparticles as compared to DPPC without any pegylation. Figure 4.3.2 shows the effects of PEG-DSPE ratio on size of DPPC and PEG-DSPE2k. With an addition of 5 mol% of PEG-DSPE2000, particles were nano-sized. Rovira-Bru *et al* showed that with the increase in PEG, the symmetric bilayer structure of DPPC becomes unstable, while the spherical structure becomes optimal. This is due to PEG being inserted to the outer monolayer, increasing the asymmetry [8].

Figure 4.3.2: Effect of PEG-DSPE ratios to size

4.4 Effect of process parameters on size – Flow rate ratio

Flow rate ratio (FRR) refers to the ratio of aqueous buffer to organic solvent in the microfluidic setup. Previous studies have indicated that an increase in FRR reduces the resulting size of liposomes due to the overall higher aqueous volume; the lipids get diluted in the aqueous solvent, thus resulting in faster mixing and decreasing the particle fusion (Ostwald ripening) [9]. The similar trend was observed from DMPC liposomes that the increase in FRR (from 3:1 to 5:1) reduces liposome size from 33nm to 29nm ($p < 0.05$) as shown in Fig 4.4 and 4.1. A higher FRR also decreases the amount of ethanol from 25 v/v% (3:1), to 16.7% (5:1). Nevertheless, lipid aggregation still occurred for phospholipids with long alkyl chain length, i.e. DPPC and DSPC shown in Figure 4.1.

Hence for effective understanding of FRR and TFR, the formulation used is 95:5 mol DPPC:PEG-DSPE2000. Figure 4.4 shows the effects of varying flow rate ratio on size. Sizes were measured pre-dialysis for similar comparison to previous chapters. 3:1 and 5:1 produced nano-sized particles as compared to 1:1, where there is an equal amount of organic solvent to aqueous phase. Just like the pure DPPC and DSPC presented in figure 4.1, aggregation also occur, likely due to the abundance in organic solvent.

Figure 4.4: Effect of FFR on size. a) FFR of 5:1 Aqueous:Organic for Saturated lipids. b) Varying FFR from 1:1 to 5:1 for 95:5 DPPC: PEG-DSPE liposomes (* = 0.05 CI)

4.5 Effect of process parameters on size – Total flow rate

Apart from FFR, TFR also affects the size of liposomes as it affects the rate of mixing in the microfluidic system. Similarly a 95:5 DPPC: PEG-DSPE ratio was used. Literature has shown that size of nanoparticles was dependent on either a rapidly mixing condition or a diluted condition [10]. Rate of mixing also corresponds to the rate of polarity change. When the rate of polarity increases, lipid molecules have lesser time before it experiences ‘supersaturation’, because organic solvents are being diffused out faster. This causes the rate of diffusion to increase, meaning lipid molecules have lesser time to interact with each other to form larger fragments before being forced to curve into a spherical structure. It is thus also possible to achieve a limit-size liposome as presented by Zhigaltsev *et al.* [11]

Figure 4.5 shows the effect of TFR on size of nanoparticles. This study proves the effect of flow rate on size, although there are no significant difference at lower flow rates.

Figure 4.5: Effect of TFR on size for liposomes fabricated with 95:5 DPPC: PEG-DSPE 2k

4.6 Stability studies

Following the results of previous studies, a stability study was conducted for both formulations of liposomes that were nano-sized. Figures 4.6 describe the stability of these nanoparticles after dialysis. Particles were kept at 4°C, but particles with FRR 5:1 showed variation in sizes. It could be due to a higher PDI of 0.3 for FFR 5:1 particles as compared to a PDI of 0.1 for 3:1 particles. As such both samples could be similar in size but a more polydisperse sample of 5:1 generated more fluctuation in measurement data.

Figure 4.6: Stability of 95:5 DPPC:Peg-DSPE2000 liposomes over 8 weeks

4.7 Summary

Results reveal that saturated lipids are unable to form stable liposomes. However visual observations showed that an optically clear solution turned turbid within minutes as it cooled to room temperature. Control experiments using thin film hydration proved that DPPC liposomes did not aggregate when the temperature decreased. It suggests that the cause of aggregation, while temperature triggered, must be in combination with another source. As microfluidic operations in this study involves large amounts of organic solvents, it was speculated that organic solvent might play a part in causing this aggregation. It is largely ignored in literature as current liposome fabrication methods do not involve organic contents that are higher than what is being used in this studied. The use of additives also seems to be playing a different role. Cholesterol is commonly used to improve stability of liposome formulations, but in this study is used to reduce the transition temperature of DPPC. Similarly, the polymer PEG widely known for its improvement to circulation half-life, is used for increasing the asymmetry of the outer monolayer in this case.

It was hypothesized here that size aggregation and instability of microfluidic liposomes are a direct consequence of the presence of interdigitation in these liposomes (Figure 5.1 a-b). Interdigitation refers to the phenomenon where two opposing leaflets of a bilayer interpenetrate into one another and form a single layer. When this happens, aggregation results as the single layer is not thermodynamically stable. Such interdigitation can be induced by pressure, chemicals or by the type of lipid structure. This warrants a mechanistic study which will be discussed in the next chapter.

For the same lipid composition, it can be observed that the higher flow rate ratio and the highest total flow rate results in liposomes with the smallest size. Solvent dilution techniques are thus affected by rate of polarity change, evident from both FFR and TFR changes.

While it is important to understand both process parameters on size, Kastner *et al* has shown using simulations that FFR has a greater importance in deciding the size. [12].

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Chapter 5 Mechanistic study of liposomes fabricated using microfluidics

In this chapter, a further study was performed on the feasibility of forming liposomes using only saturated lipids. It was well explored previously that liposomes could not successfully form, even if process parameters were varied. DMPC was the only saturated lipid that could form liposomes at room temperature. A temperature induced aggregation was considered and it was also visually observed that the solution turned optically turbid within minutes. Here, a differential calorimetric test, together with advanced x-ray techniques were used to examine the effects of interdigitation, which might be the cause of the aggregation.

5.1 Effect of solvent type on liposome formation

In the previous chapter, it was understood that transition temperature and the aid of additives seem to be preventing lipid aggregation for saturated lipids. However the exact mechanism was unknown, as well as the temperature at which this aggregation occur. One likely phenomenon is interdigitation, where the phospholipid alkyl chains merge, and the bilayer flattens. It could be due to pressure, structure of the lipid or chemical compounds. In this case, organic solvent is the chemical compound that is likely to be causing interdigitation.

The effect of organic solvent type on liposomal formation by microfluidics using methanol was then studied. Figure 5.1 shows a schematic of interdigitation and DLS measurement data for saturated lipids in MeOH. As shown in Figure. 5.1 c-d, the same trend was observed with only DMPC forming liposomes. It was hypothesized that this trend happened due to the interdigitation of the phosphatidylcholines induced by short-chain alcohols. It was well established that saturated-chain PCs particularly DPPC and DSPC, can form interdigitated structure phases, in which the acyl chains extend beyond the bilayer mid-plane and interpenetrate into the opposing monolayer Figure. 5.1 a-b, in the presence of short-chain alcohols (methanol or ethanol) when the temperature is lowered below the T_m of the phospholipid. When interdigitation proceeds, it produces deformed vesicles that have membrane defects where hydrophobic domains are exposed. The liposomes may fuse together at these hydrophobic defects into large extended planar sheets of interdigitated phospholipid. This is often observed for saturated phospholipids with the trans configuration [1].

Figure 5.1: Schematic diagram showing the conversion of saturated PCs from the (a) non-interdigitated to the (b) fully interdigitated phase when the ethanol concentration is above its threshold concentration for the formation of the fully interdigitated phase.

Z-average of saturated lipids in Methanol (c) 3:1 Aqueous:Organic Flow rate ratio (d) 5:1 Aqueous:Organic Flow rate ratio

5.2 Effect of ethanol concentration on liposome formation

Generally, DPPC does not spontaneously interdigitate when hydrated; however, it can be chemically induced into interdigitated phase in the presence of ethanol leading to lipid aggregation, as observed in the current microfluidics study. To better illustrate the influence of ethanol on liposome aggregation and/or fusion, the conventional thin film hydration method was used to fabricate liposome by hydrating the lipid thin film with various amounts of ethanol. Figure. 5.2a showed changes in particle size of DPPC liposomes fabricated using thin film hydration as a function of temperature at a variety of ethanol concentrations. In the absence of ethanol, there was no apparent particle size changes as the temperature decreased. Above 25% ethanol concentration, however, the apparent size changes were slow when the temperature was above the transition temperature of DPPC (i.e. 41°C) and the increment in the DPPC particle size was abrupt when the temperature decreased below the T_m , indicating large aggregates formed in the suspensions. It was

previously shown that membranes of DPPC could readily form interdigitated structure in the presence of ethanol when the ethanol concentration is above its threshold amount (1.1 M) for a lipid concentration of 5 mg/ml; moreover the typical alcohol content used in microfluidics (10 – 25% or 1.92 – 4.8 M) is well above the threshold concentration for a lipid concentration of 3 mg/ml.

In the presence of such high ethanol concentration, the water molecules associated with hydrophobic groups of Phosphatidylcholine (PC) are replaced with ethanol molecules, which adsorb on the membrane surface or distribute into the interfacial region between the hydrophobic and hydrophilic part of membranes [2]. As ethanol is a larger molecule than water, this increases the lateral separation between the phospholipid head groups, creating energetically unfavorable voids. When the temperature is above the T_m of DPPC, the hydrocarbon chains are in the fluid state and therefore can accommodate the increased lipid head group separation produced by the presence of ethanol. However, when the temperature is decreased below the T_m , this increase in head group volume instead leads to hydrocarbon chain tilting and sliding from opposing bilayers past each other. This displacement then induces DPPC interdigitated membrane formation and aggregations due to the disappearance of highly hydrated water on the bilayer surfaces.

Since interdigitation is highly unfavorable in the liquid crystalline phase (above T_m), further investigation was performed to see if removal of ethanol above T_m of PC would prevent aggregation of DPPC liposomes. As such, dialysis was performed above T_m of PC (i.e. 60°C) overnight for ethanol removal. To confirm successful ethanol removal, the residual solvent level was measured using gas chromatography. Result indicated that the residual solvent amount is less than 0.2% after dialysis and it is below ICH guidelines of less than 3000 parts per million, to ensure regulatory compliance [3][4]. Figure 5.2b depicted the particle size changes as a function of temperature before and after dialysis of DPPC liposomes fabricated using microfluidics. In the presence of ethanol without dialysis, the apparent size change was slow when the temperature was above the transition temperature of DPPC (i.e. 41°C) and the increment in the DPPC particle size was abrupt when the temperature decreased below the T_m , indicating large aggregates formed in the suspensions. Interestingly, after ethanol removal by dialysis, there was no apparent particle size changes as the temperature decreased. Similar results were obtained with another saturated phosphatidylcholine DMPC as shown in Figure. 5.2c. Significant particle size change occurred when the temperature was dropped below the T_m of DMPC (i.e. 24°C) in the presence of ethanol

without dialysis. These results indicate that presence of high ethanol content in microfluidics causes the lipid interdigitation below the transition temperature region and ethanol removal above the T_m could prevent the formation of interdigitated structures and subsequent aggregated states, retaining the nano-vesicle structures (Figure. 5.2d).

Figure 5.2 :Changes in apparent Z-average diameters of DPPC liposomes fabricated by film hydration as a function of temperature at a variety of ethanol concentrations. Comparison of particle size as a function of temperature for (b) DPPC and (c) DMPC liposomes fabricated using microfluidics before and after dialysis. Dialysis was performed above the T_m of respective PCs overnight. (d) Cryo-TEM images of DPPC liposomes after dialysis.

5.3 Cholesterol mitigates effect of interdigitation

As well described by literature [5], [6], the incorporation of cholesterol to the DPPC membrane helps to stabilize the bilayer and prevent interdigitation formation above the alcohol threshold concentration. To further investigate this phenomenon, the inhibiting effect of cholesterol (10 to 50 mol%) on DPPC liposome production using microfluidics was explored. In Figure 5.3a, increasing cholesterol content reduces liposome size from approximately 500 nm (25 mol%) to 100 nm when equimolar DPPC: Chol was employed. However, only DPPC: Chol liposomal formulation containing 50 mol% cholesterol is highly monodisperse ($PDI = 0.07$), indicating the interdigitation-driven aggregation was completely inhibited. The inhibiting effect by the addition of cholesterol in PC bilayers is due to the cholesterol alleviates the lipid head-group crowding that favors interdigitation. Previously, Komatsu and Rowe showed that cholesterol prevents interdigitation in the presence of 20 mol% cholesterol [7], and in this present study, results showed that 25 mol% cholesterol added would prevent the onset of interdigitation and 50 mol% cholesterol for complete interdigitation inhibition. This finding is consistent with the model suggested by Copeland and McConnell [8], assuming the regions of pure DPPC become interdigitated and the region containing 25 mol% cholesterol do not. This could lead to highly poly-disperse liposomal formulation with $PDI = 0.9$. Moreover, the result is consistent with Forbes *et al.*[9], who demonstrated that nano-liposome formation was obtained using microfluidics at 25 °C when 50 mol% cholesterol was incorporated in DSPC liposomes at a similar lipid concentration of 10 mg/mL.

Furthermore, with the inclusion of 50 mol% cholesterol, the production of DPPC: Chol liposomes using microfluidics can be performed at room temperature, alleviating the stability issues for incorporating thermally-labile drugs and proteins. The vesicular structure was also maintained pre and post dialysis (Figure 5.3b-c). One possible reason is that as cholesterol amount is increased to 50 mol%, the gel-to-liquid phase transition disappears as shown by DSC. It could mean that at such high cholesterol content, the system exists as a liquid disordered phase throughout facilitating liposomal formation using microfluidics at room temperature.

Figure 5.3 : (a) Particle size and size distribution for DPPC-Cholesterol liposomes fabricated using microfluidics at FRR 3:1 and 12 ml/min TFR at room temperature. Molar ratio is DPPC: Cholesterol and DPPC concentration set at 18 mM. Cryo-TEM images of liposomes with 50:50 mol% DPPC: Cholesterol (b) before dialysis and (c) after dialysis.

5.4 Differential scanning calorimetry of DPPC and DMPC

Previous results demonstrate that high ethanol concentrations used in microfluidics can induce aggregation formation due to lipid interdigitation in saturated phosphatidylcholines, especially for the saturated PCs with high transition temperature. The presence and extent of interdigitation using DSC and SAXS was further investigated. To determine the thermodynamics of interdigitation, DSC is often used. Using DSC, sensitive monitoring the T_m hysteresis of heating and cooling curves, characteristic of interdigitation. The hysteresis is due to the irreversible energy loss as ethanol molecules bind to and leave the saturated phospholipid layers. Apart from the hysteresis, the suppression of a pre-transition peak is another characteristic of alcohol-induced interdigitation of saturated PCs. This is because when ethanol has bound to the bilayer, there is no transition from ordered gel phase to an ordered fluid phase. By comparing the DSC curves of microfluidic samples before and after dialysis (Figure 5.4.1 a and b), the emergence of T_m hysteresis and absence of pre-transition peak in the presence of ethanol was observed, suggesting the existence of alcohol-

induced interdigitation. Once the ethanol is removed via dialysis, the DSC thermogram of DPPC exhibits the typical thermodynamic profile, showing the pre-transition from the planar gel to the ripple gel phase and the absence of transition hysteresis (Figure 5.4.1b). It illustrates the reversibility of DPPC phase transition in the absence of ethanol.

To confirm that lipid interdigitation formation is not related to the manufacturing process, DPPC liposomes with ethanol content above threshold limit were fabricated using conventional film hydration. Film hydrated samples with a mixture of organic solvent and PBS were compared against neat PBS (Figure 5.4.1c-e). The DSC thermograms of film hydrated samples were similar to that of the microfluidic's calorimetry. The presence of transition hysteresis and suppression of pre-transition peaks both suggested that interdigitation occurs in both microfluidic and film hydrated samples, as long as the ethanol content is above the threshold limit of 1.1 M.

In the previous sections, DMPC liposomes were successfully fabricated using microfluidic processes. However, it was hypothesized that the same phenomenon of aggregation will occur in DMPC at ethanol concentrations above the threshold concentration. Figure 5.2c shows the abrupt change in size when the formulation containing ethanol falls below the transition temperature. Indeed, Figure 5.4.2 a-b showed that the microfluidic fabricated liposomes have both the signature characteristics. Figure 5.4.2 c-e also showed similar traits to the calorimetry of DPPC liposomes fabricated using thin film hydration, with only a slight change in the peak location.

Figure 5.4.1: Heating (red lines) and cooling (blue lines) DSC thermograms of DPPC liposomes before and after dialysis. The direction of exothermic reaction is pointed downwards. Samples were left to equilibrate at room temperature before initializing measurement. (a) Microfluidics before dialysis, (b) microfluidics after dialysis, (c) thin film hydration in PBS, (d) thin film hydration in 75:25 PBS:EtOH before dialysis, and (e) thin film hydration in 75:25 PBS:EtOH after dialysis.

Figure 5.4.2: Heating (red lines) and cooling (blue lines) DSC thermograms of DMPC liposomes before and after dialysis. The direction of exothermic reaction is pointed downwards. Samples were left to equilibrate at room temperature before initializing measurement. a) Microfluidics before removal of ethanol through dialysis, b) microfluidics after removal of ethanol through dialysis, c) thin film hydration in PBS, d) thin film hydration in 75:25 PBS EtOH before dialysis, and e) thin film hydration in 75:25 PBS EtOH after dialysis.

5.5 Small angle X-ray Scattering for DPPC, DMPC and DPPC/Chol

Since DSC can detect the interdigitation of lipids, it is crucial to understand the effects of interdigitation and how the lipid bilayer is affected. To further investigate the organization of DPPC membranes in the presence of ethanol, SAXS measurements were performed. X-ray scattering patterns at small angles yielded information on long range bilayer organization and the d-spacing of SAXS represents repeat distance of multi-bilayers, including the thickness of the bilayer (i.e. the distance between two phosphate groups of the opposing bilayer surfaces) and that of water layer. The diagrams in Figure 5.5 show the scattering signals of DPPC samples before and after removal of ethanol using a low noise flow cell detector unit.

For the DPPC samples without ethanol in Figure 5.5a after dialysis, the value of d-spacing obtained is 63 Å, which is consistent with previously reported d-spacing values of DPPC bilayers [10]. In contrary, the d-spacing is 49 Å for DPPC samples containing 25 v/v% ethanol before dialysis. The lower value of the d-spacing is an indication of interdigitation. In the presence of ethanol, the hydrophobic acyl chains are inserted into the opposite leaflet. The difference in d-spacing between the fluid non-interdigitated and interdigitated samples reported previously was found to be approximately 11 Å [11], which is similar to the findings of this current study. Additionally, DMPC, another saturated PC, was investigated to validate the chemically induced interdigitation phenomenon. As shown in Figure 5.5b, the d-spacing values of 41.8 Å and 63.8 Å are obtained for DMPC sample before dialysis and after dialysis, respectively. This finding is consistent with Kučerka *et al.*, in which a similar d-spacing of 62.6 Å was obtained for non-interdigitated DMPC liposomes [12]. The increase in d-spacing after removal of ethanol suggests that interdigitation formed using microfluidics is reversible, which is an interesting finding, considering there are limited literature on reversibility of interdigitation.

Figure 5.5: Small angle X-ray Scattering of various lipid formulations before and after removal of ethanol. Graphs representing after dialysis was arbitrary raised to show contrast. (a) DPPC microfluidics, (b) DMPC microfluidics, (c) 50:50 DPPC: Cholesterol microfluidics and (d) 50:50 DPPC: Cholesterol thin film hydration.

Moreover, it has been understood that cholesterol prevents interdigitation by orientating itself near the headgroup of the phospholipid, preventing interaction of ethanol and DPPC [13]. Cholesterol interacts near the interfacial area of the bilayer. As cholesterol intercalates between the lipid headgroups, it increases the area per headgroup, reducing steric destabilization that leads to interdigitation. Another possibility is that since cholesterol must not be interacting with water molecules; the smaller bilayer thickness of the interdigitated structure cannot accommodate it; therefore, the interdigitation structure cannot be formed. In general, cholesterol pulls the equilibrium towards a non-interdigitated phase [14]. Figure 5.5c and d show the small angle scattering signals of DPPC: Cholesterol (1:1 mol ratio) samples fabricated using microfluidics and thin film hydration, respectively. Both samples before and after removal of ethanol show the

similar d-spacing of 63-65 Å. The scattering signal of DPPC: Cholesterol shows that there are negligible differences ($p < 0.013$) between the d-spacing before and after dialysis. The result of this study is consistent with various studies of how cholesterol prevents interdigitation [13], [14].

5.6 Role of interdigitation in microfluidic-directed liposome self-assembly

Taken together, Figure 5.6.1 schematically describes the details of the DPPC liposomal system fabricated using microfluidics. In microfluidic processing, it is observed that DPPC undergoes large size changes when it is processed below its main transition temperature. However, removing ethanol above its T_m while maintaining its liquid disordered structure retains the vesicle size. Furthermore, the addition of equimolar amounts of cholesterol allows liposome formation at temperatures below its original transition temperature of 41 °C. The novelty of this study shows that in phospholipids having an interdigitated system caused by the presence of ethanol, it can be prevented either by the cholesterol incorporation, or reversed by ethanol removal above T_m . This is especially critical in microfluidic systems, wherein the organic solvent is often used beyond the threshold limit of interdigitation.

Figure 5.6.1: Schematic of DPPC interdigitation in the presence of ethanol. Ethanol causes chain tilt and favourable interdigitation of DPPC lipids above ethanol threshold limit of 1.1 M (i.e. 6.6 v/v%). Incorporation of cholesterol to DPPC formulation avoids interdigitation through preventing chain tilt induced by ethanol [15].

5.7 Summary

The study in this chapter demonstrated that for interdigitation to occur, operating temperature should be below the lipid transition temperature, as well as having an organic solvent content greater than the threshold of the lipid. In this study, the lipid transition temperature is 41°C and the ethanol concentration was 1.1M. Lipid interdigitation was not due to a difference in fabrication, as thin film hydrated liposomes also underwent the same phenomena. Interdigitation caused a decrease of d-spacing of approximate to 13Å, or 1.3nm for DPPC. It is crucial to understand this phenomenon, because interdigitation leads to undesired aggregation and fusion.

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Chapter 6 Drug encapsulation study for hydrophilic and hydrophobic drugs using Microfluidics

In this chapter, the encapsulation capacity of microfluidic fabricated liposomes was studied. A hydrophobic drug Latanoprost and hydrophilic model drug Dextran-FITC were chosen for the study. The aim was to examine the effect of fabrication methods, lipid and process parameters on the encapsulation efficiency of liposomes. It was hypothesized that microfluidic fabrication of liposomes are able to encapsulate payloads to the same extent of conventional thin film hydration. For the hydrophilic model drug, different charges and molecular weights were evaluated for encapsulation capacity. As it was conventionally difficult to load small hydrophilic drugs [1], the aim was to verify if microfluidics was suitable, and if the presence of charge or molecular weight would improve the encapsulation efficiency.

Study of hydrophobic drug Latanoprost

6.1 Effect of lipid formulation on encapsulation efficiency

Latanoprost, an ester prodrug used for lower intraocular pressure, was used in this study. It is a small hydrophobic drug, of molecular weight 432 g/mol.

To investigate the role of lipid composition for the encapsulation efficiency of latanoprost, different lipid compositions were considered (Table 6.1). The lipid compositions were chosen based on considerations in previous Chapters 4 and 5. Liposomes were produced at 1.5:1 FRR and 2ml/min TFR with operating temperature at 60°C. The liposome size and the corresponding encapsulation efficiency were investigated after solvent removal, as shown in Table 6.1.

Table 6.1: Physical properties of Latanoprost loaded liposomes using Microfluidics

Formulation at 9mM using MF	Size (nm)	EE (%)	PDI
DPPC	48.9 ± 5.45	23.3 ± 0.8	0.22 ± 0.006
DPPC Cholesterol (50:50)	104.0 ± 0.73	0.6 ± 0.2	0.12 ± 0.014
EPC	99.5 ± 3.80	44.6 ± 6.5	0.25 ± 0.006
EPC:Chol (50:50)	122 ± 9.40	13.8 ± 0.5	0.24 ± 0.023
POPC	99.5 ± 1.47	46.2 ± 6.7	0.25 ± 0.018
EPC Peg-DSPE2000 (95:5)	73.2 ± 0.64	49.8 ± 6.4	0.20 ± 0.005

Effect of Cholesterol on encapsulation efficiency

As described in previous chapters, the incorporation of cholesterol helps to stabilize the bilayer and prevent interdigitation, even in the presence of organic solvents. The incorporation of 50 mol% cholesterol allows fabrication of such liposomes at room temperature. This proves to be immensely important in allowing saturated lipids to form liposomes using microfluidics.

Since DPPC liposomes could be fabricated by incorporating cholesterol, the logical step was to investigate the ability to carry payloads. Despite the benefits of using cholesterol described previously, cholesterol inhibits hydrophobic drug from being loaded into the bilayer, simply by occupying the interfacial area [2]. This results in lower drug encapsulation, especially for hydrophobic drugs. From Table 6.1, DPPC with cholesterol only managed to encapsulate 0.6% of Latanoprost, compared to DPPC, which was 23%. Also, the encapsulation efficiency of EPC-Cholesterol (50:50) was also lower than using EPC alone (13.8% vs 44.6%). Previously, Deniz *et al.* [3] reported a reduction of 30% in EE with the incorporation of cholesterol in a liposome model made using thin film hydration. In this study, the extent of cholesterol reducing encapsulation efficiency was approximately 20% for DPPC, and 30% for EPC. This result is also consistent with the findings of Smondyrev *et al.*, that presented a simulation model where a 45 mol% cholesterol would decrease the area per DPPC from $64.5\text{\AA}^2$ to $42\text{\AA}^2$, which is approximately 35% reduction in volume that could be used to retain Latanoprost. [4], [5]. This could mean that while cholesterol helps maintaining the bilayer structure in a liquid disordered state, there is lesser space for the drug to be retained in.

Saturated lipids and unsaturated lipids

Saturated lipids such as DPPC do not contain double bonds within their hydrophobic tails, whereas unsaturated lipids contain one or more double bonds within their hydrophobic tails. It is well understood that these difference results in transition temperature, chain fluidity and packing density differences [6], [7].

The results showed that encapsulation efficiency of EPC (44.6%), POPC (46.2%) and EPC:PEG-DSPE(2000) (49.8%) was twice of that of DPPC (23.3%). The differences in the EE could be attributed to the packing density differences. Kučerka *et al.* [8] showed that the volume in hydrophobic tail for an unsaturated lipid was 14.5% greater than a saturated lipid with similar chain length, at room temperature. As the volume in the hydrophobic tail is lower in DPPC than EPC, it is anticipated that less drug would be embedded in DPPC liposomes.

6.2 Effect of process parameters and fabrication method on drug release

Previous results (Figure 4.2) demonstrated that unsaturated lipids such as EPC and POPC are able to form liposomes using microfluidics simply, as they are not affected by interdigitation like the saturated lipids. The effect of process parameters on drug encapsulation was further investigated. From previous experiments, a change in FRR results in a smaller size. Size of 3:1 FRR liposomes are statistically lower than 1.5:1. The size for liposomes of 5:1 FRR was expected to be lower than 3:1, but the liposome size was large and size distribution was heterogenous with PDI = 0.5 (Fig 6.2.1). It could be due to high solvent polarity difference between a low amount of organic solvent and high amount of aqueous buffer. This could lead to instability during the nucleation process, resulting in aggregates. López *et al.* [9] have also discussed the effects of solvent polarity from high FRR that might result in a more polydisperse population.

Table 6.2 showed that encapsulation efficiency was not affected by the change in process parameters between 1.5:1 to 5:1 (Aqueous: Organic). In encapsulation of a hydrophobic drug, the partition coefficient between the drug and the lipid plays an important role. The partition coefficient is a concentration ratio of drug between the lipid bilayer and aqueous phase. It is interpreted as the ability/affinity for the bilayer to entrap/diffuse the drug, and it is a function of lipid characteristics and drug/lipid ratio. In a previous study by Natarajan *et al* [10], partition coefficient was experimentally determined for a drug/lipid ratio of 0.1. The same drug/lipid ratio was used in this study. The change in process parameters does not change the partition coefficient so the same capacity for the bilayer to carry the drug was anticipated. As a result, there should not be a difference in encapsulation efficiency between process parameters. An extension of this

hypothesis suggests that fabrication methods should not affect partition coefficients, as long as lipid characteristics and drug/lipid ratio was maintained. To prove the hypothesis that fabrication methods do not affect encapsulation efficiencies, two control studies were performed using thin film hydration. The first control study was thin film hydration method in which the rehydration was performed using pure PBS while the second control study used 60:40 PBS:EtOH as the rehydration medium. This was to re-enact the solvent condition in microfluidics. The drug release of latanoprost was based on the diffusion out of the bilayer to the aqueous phase, which also depended on the partition coefficient. As mentioned above, since the partition coefficient was assumed to be consistent despite the difference in fabrication method, a similar drug release profile was also anticipated. Figure 6.2.2 showed that over 28 days, all formulations tested presented a first order kinetics release. The drug release further proves the congruence of microfluidics and thin film hydration for the loading of Latanoprost, a hydrophobic small molecule drug.

Figure 6.2.1: Z-average of Latanoprost loaded EPC fabricated using Microfluidics and Thin-Film Hydration

Figure 6.2.2: Cumulative (%) drug release profile of Latanoprost loaded EPC liposomes over time

Table 6.2: Encapsulation efficiency of Latanoprost loaded EPC fabricated using Microfluidics and Thin-Film hydration

EPC FFR &TFR at 9mM	Size (nm)	PDI	Size after 20 days (nm)	EE (%)
1.5:1 2ml/min	99.5 ± 3.8	0.2 ± 0.006	101.1 ± 2.7	44.6 ± 6.5
3:1 2ml/min	82.7 ± 2.9	0.16 ± 0.005	84.8 ± 6.0	50.8 ± 2.0
5:1 2ml/min	176.6 ± 25	0.52 ± 0.025	-	51.1 ± 3.0
Thin film (PBS)	110.9 ± 1.5	0.06 ± 0.010	112 ± 0.3	46.5 ± 1.8
Thin film (60:40 PBS:EtOH)	89.3 ± 1.0	0.3 ± 0.002	100.2 ± 0.8	49.7 ± 5.0

6.3 Study of hydrophilic model drug Dextran-FITC

6.3 Effect of lipid formulation on encapsulation efficiency of small MW Dex-FITC

It was observed that the hydrophobic drug loading profile was similar for both Microfluidics and Thin-Film hydration. Following this, it is important to understand the drug loading behaviour of hydrophilic drugs. In microfluidics, lipids and drugs are brought very close together upon mixing, whereas thin film hydration involves rehydration of a lipid film with the aqueous medium containing the drug. There is lesser interaction during rehydration between the bilayer and the drug, owing to their hydrophobic and hydrophilic nature respectively; the partition coefficient does not play a critical role in hydrophilic drug loading. One of the interactions that could be manipulated was the hydrophilic headgroup and the hydrophilic drug. To improve the interaction between the hydrophilic headgroups of lipids and the drug, charged lipid/drug variants were studied in this chapter.

Table 6.3a showed the type of charged/neutral lipid and drugs that were used in the study. The results in Table 6.3b showed that the loading of Dex-FITC 4k was low, even with the inclusion of charged lipids or using charged variants of Dex-FITC. Although there is favourable interaction between the cationic lipid and anionic drug, encapsulation efficiency was below 10%. It was possible that the low encapsulation of small molecule Dextran-FITC was due to the inability of the liposome to retain the drug within the aqueous core. Figure 6.3.1 shows a schematic of possible drug-leakage between varying molecular weights of Dex-FITC. Table 6.3c shows an approximate radius of the Dex-FITC molecule for varying molecular weights. A typical EggPC bilayer has a pore size of 30 Å [11]. With Dex-FITC 4k of stokes radius of 1.4nm and a pore size of EggPC of 3 nm, it was expected that EggPC liposomes would be unable to retain Dex-FITC 4k. Bozzuto *et al.* [12] have published an extensive review on how different types of hydrophilic drugs are able to escape the carrier systems, resulting in poor encapsulation efficiency, or burst release.

Table 6.3a: Lipids and Dextran-FITC drugs combination pair

Hydrophilic model drugs	Lipids
Dextran FITC mw 4k (neutral)	EPC (neutral
Dextran FITC mw 150k (neutral)	EPC:Dotap:Cholesterol (EPC/DOTAP/Chol) (6:2:2) (Positively Charged)
Dextran Carboxymethyl mw 4k (Negatively Charged)	
Dextran Carboxymethyl mw 150k (Negatively Charged)	

Table 6.3b: Physical properties for Lipids and Dex-FITC 4k microfluidics. BC- Before centrifugation. AC- After centrifugation.

Lipid	Drug (4k)	Size BC (nm)	Size AC(nm)	PDI	Zeta Potential (mV)	EE (%)
EPC (neutral)	Neutral	118 ± 0.4	108 ± 0.4	0.22 ± 0.005	-1.0 ± 0.7	4.2 ± 0.9
EPC (neutral)	Charged	114 ± 1.6	101 ± 0.8	0.21 ± 0.006	-0.76 ± 0.1	4.1 ± 0.2
EPC/DOTAP/Chol (charged)	Neutral	98 ± 0.5	94 ± 0.4	0.23 ± 0.007	15.4 ± 1.5	9.5 ± 0.5
EPC/DOTAP/Chol (charged)	Charged	99 ± 1.2	90 ± 1.4	0.21 ± 0.022	13.7 ± 0.5	9.4 ± 0.5

Table 6.3c: Radius of Dextran corresponding to its Molecular weight for drug leakage predictions.

Molecular Weight (MW) of Dextran (Dalton)	Approximate Stokes' Radius (Angstrom)
4,000	14
150,000	85

Figure 6.3.1: Schematic of aqueous core leakage from a liposome with credit from Encapsula

6.4 Effect of lipid formulation on encapsulation efficiency and release of large MW Dex-FITC using Microfluidics

To understand if the size of the hydrophilic drug is the cause of rapid escape from liposome, a higher molecular weight of Dex-FITC 150k was used. Table 6.4a shows the encapsulation of liposomes loaded with Dex-FITC 150k. Comparing the charged Dextran-FITC with both types of charged/neutral lipids (row 2&4, Table 6.4a), drug encapsulation improved with the use of charged lipid DOTAP. It was hypothesized that the anionic drug could interact electrostatically with the lipid during nano-precipitation, it could be bound within the aqueous core and the presence of

charge would reduce its chance to escape. Hence the encapsulation improved to 51.6% from 7.6% when the lipid was charged. This result is in-line with the study from Torchilin *et al* [13], where the presence of positively charged lipids in a liposome allowed for improved encapsulation of DNA and efficient cell transfection. It was also possible that the charged drug at 150k repelled other drugs in the aqueous medium, and the lack of interaction between the neutral lipid and charged drug resulted in a low encapsulation.

It was observed that there was unusually high encapsulation for neutral Dextran-FITC in both lipid types used. Since there was no interaction between the lipids and neutral drug, it was anticipated that encapsulation efficiency of the neutral drug in both lipid variants (65% for charged lipid and 66% for neutral lipid) to be lower than charged drug/lipid pairing (51%). The high molecular weight of the Dex-FITC might have allowed the drugs to be loosely bounded to the surface of the liposomes, where hydrophilic groups have an interaction with. To verify this, a drug release was performed and measured until it reached a plateau, where the ‘burst’ release in the initial hours (0-24hr) were considered loosely bounded drugs and the plateau referred to drugs trapped within the aqueous core of the liposomes. The remaining drug content would be evaluated as truly encapsulated within the aqueous core of the liposomes. These liposomes were then harvested and broken followed by the drug content quantification. Also, drug released and encapsulated were tallied to ensure 100% recovery. Figure 6.4.1 showed the cumulative drug release profile for Dex-FITC 150k loaded liposomes. From the 3rd hour, there was a statistical difference between the release of neutral drug/lipid pair and charged drug/lipid pair. After 24 hours, the plateau was observed. The remaining liposome solution were harvested and broken to analyse the true drug encapsulation. Table 6.4b showed the true encapsulation of Dex-FITC 150k for both neutral and charged drug/lipid pairs. It was calculated by multiplying the amount of drugs present before release was performed, with the final drug contents left inside liposomes after the plateau was established. For example, neutral drug/lipid pair had an initial drug content of 66% before release was performed, and a final drug content of 30% in the liposomes after the plateau was established. Hence, the true encapsulation was the 30% of 66% drug present initially, which is 18.5%. For the charged lipid/drug, the true encapsulation was 25.6%. It was after removing unbound drug and observing the release profile, that the presence of charge does improve drug encapsulation. This verifies the initial hypothesis, that electrostatically favourable interactions improve drug encapsulation.

Table 6.4a: Physical properties of Dex-FITC 150k loaded liposomes using microfluidics

No.	Lipid	Drug (150k)	Size Before centrifuge (nm)	Size After centrifuge (nm)	PDI	Zeta Potential (mV)	EE (%)
1	Uncharged	Uncharged	115 ± 0.9	151 ± 0.4	0.21 ± 0.015	0.2 ± 0.448	66.0 ± 0.5
2	Uncharged	Charged	119 ± 1.9	121 ± 0.8	0.22 ± 0.003	0.1 ± 0.025	7.6 ± 2.1
3	Charged	Uncharged	120 ± 0.6	123 ± 0.5	0.21 ± 0.019	13.9 ± 0.200	65.0 ± 1.7
4	Charged	Charged	119 ± 0.4	113 ± 1.4	0.22 ± 0.020	12.2 ± 0.435	51.3 ± 1.2

Figure 6.4.1: Cumulative drug release profile of Dex-FITC 150k loaded liposomes using microfluidics**Table 6.4b:** True encapsulation efficiency of Dex-FITC 150k using microfluidics

Lipid	Drug (150k)	True encapsulation (%)
Uncharged	Uncharged	18.5 ± 3.9%
Charged	Charged	25.6 ± 2.6%

6.5 Effect of fabrication methods on encapsulation efficiency and drug release of large MW Dex-FITC

Fundamentally, thin film hydration and microfluidics operate differently, with film hydration being a top down process and microfluidics being a bottom-up approach. This does not affect how a hydrophobic drug, Latanoprost, reside within a liposome. The reasons were explained prior in Section 6.2. The effect of fabrication method on the encapsulation of a large molecule hydrophilic drug was important in understanding if there are any drug encapsulation benefits of using microfluidics apart from being time-saving and ease of industrial scaling-up.

Table 6.5a shows the physical properties for uncharged and charged liposome pairing manufactured by thin film hydration. Both the encapsulation efficiencies were lower than the microfluidic counterpart. A detailed schematic for the two fabrication methods was shown in Figure 6.5.1.

During a thin film hydration process, the lipid film was prefabricated before the aqueous medium containing the hydrophilic drug was added. For the thin film hydration setup, lamellar structures beneath the topmost layer are often excluded from the interaction with the hydrophilic drug. Most of the hydrophilic drugs will remain in the bulk aqueous phase and only a small amount of drugs can penetrate or diffuse through the MLV. This is due to low diffusivity of the hydrophilic drug through the hydrophobic bilayer. During extrusion, although the MLV are slowly broken into smaller LUVs, the aqueous volume that the liposome occupy would still be much lesser than the

bulk hydration medium, where most of the hydrophilic drug resides. As passive loading does not have driving force for drugs to diffuse towards the aqueous core, most of the liposomes would remain devoid of the hydrophilic drug, as there was already little interaction during the initial rehydration. As a result, encapsulation efficiency is often low for liposomes fabricated using thin film hydration, with a passive loading method [14].

In microfluidics, the lipids and aqueous phases were brought in proximity during mixing under the presence of electrostatic attraction for charged entities. This attraction together with the proximity of lipid and drugs may improve the fate of drug being encapsulated in the aqueous core of the liposomes. The single step liposome fabrication process also prevents drugs from escaping the bilayer unlike distortions caused to it in the case of extrusion method. Higher encapsulation in microfluidics was a result of in-situ liposome production in a single step. The use of ethanol in the organic phase could have also helped make the bilayer more flexible towards encapsulating Dex-FITC [15]. Elsana *et al.* concluded that microfluidic method was more suitable than film hydration for the encapsulation of cyclodextrin for the reasons explained above. Experimental results concur with their finding, and it was suggested that even more of the drugs were loosely bounded on the surface of the liposomes made from thin film hydration, in comparison to the microfluidic fabrication process in section 6.4. In order to capture the true encapsulation efficiency, a drug release was also performed to observe the burst and plateau of the profile. The cumulative drug release profile in Figure 6.5.2 showed that the drugs, both uncharged and charged, experienced a burst profile within 10 hours. The plateau could not be observed as most of the drugs eluted within 10 hours, and subsequent values were below detection limit. It was likely that this burst was a result of drugs diffusing from the surface of the liposomes, and not the aqueous core. Like section 6.4, the remaining liposomes were harvested and broken to ensure 100% recovery. Table 6.5b showed the true encapsulation efficiency of the liposomes fabricated using thin film hydration. Both charged and neutral drug/lipid pair showed similar encapsulation efficiencies, as well as being lower than microfluidics. As the lipids were having lesser interactions with the drug owing to the fabrication method, the lower encapsulation efficiency was anticipated. Most of the drugs remained on the surface of the liposomes and diffused quickly to the bulk medium during drug release. As the fabrication method decreased the interaction between the lipid and the drug, the effect of charge between them became minimal, hence the encapsulation efficiencies were similar.

To verify that microfluidics did improve the interaction of lipids and drugs leading higher encapsulation, a second centrifugation was performed. A second centrifugation could also remove loosely bounded drugs on the surface of liposome. This could telegraph an encapsulation efficiency that is closer to its true encapsulation efficiency. The results were shown in Table 6.5c. After a second centrifugation, it was clear that encapsulation efficiency for both liposome formulations fabricated in thin film hydration were lower than that fabricated in microfluidics.

Hydrophilic drugs do not interact well with the bilayers, hence the effect of charge promoting interaction becomes increasingly important. In a microfluidics method, this effect of charge was promoted because the lipid and drug were brought close together during mixing. In a thin film hydration method, the same effect was minimized due to the lack of interaction between drug and lipids during rehydration. Effect on drug release profile and true encapsulation efficiency proved that microfluidics was the superior fabrication method for encapsulation of large molecule Dex-FITC.

Table 6.5a: Physical properties of Dex-FITC 150k loaded liposomes using Thin film hydration

Lipid	Drug (150k)	Size (BC)	Size (AC)	PDI	Zeta	Zeta After Release	EE (%)
Uncharged	Uncharged	118 ± 0.4	121 ± 2.0	0.114 ± 0.005	-1.0 ± 0.2	-1.2 ± 0.4	31 ± 1.7
Charged	Charged	103 ± 0.8	114 ± 0.2	0.07 ± 0.013	12.3 ± 1.0	9.9 ± 0.7	33 ± 0.6

Table 6.5b: True encapsulation of Dex-FITC 150k using thin film hydration

Lipid	Drug (150k)	True encapsulation (%)
Uncharged	Uncharged	8.0 ± 2.4%

Charged	Charged	$7.2 \pm 0.2\%$
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Table 6.5c: Effect of first and second centrifugation on encapsulation efficiency of Dex-FITC 150k-loaded liposomes manufactured by thin film hydration method and Microfluidics.

Lipid	Drug (150k)	EE After 1 st UC (%)		EE After 2 nd UC (%)	
		Film hydration	Microfluidics	Film hydration	Microfluidics
Uncharged	Uncharged	31 ± 1.7	58 ± 0.03	7.2 ± 0.5	40 ± 0.5
Charged	Charged	33 ± 0.6	47 ± 0.6	7.3 ± 1.4	36.5 ± 0.1

Figure 6.5.1: Schematic of thin film hydration (left) and microfluidics (right) fabrication process. Thin film hydration is usually followed by an extrusion to get large unilamellar vesicles (LUV). Microfluidics fabricated LUVs in situ.

Figure 6.5.2: Cumulative drug release profile of Dex-FITC 150k-loaded liposome manufactured by thin film hydration method

6.6 Summary

The results in this chapter demonstrated the efficiency of loading a hydrophobic model drug Latanoprost into liposomes fabricated with both microfluidics and thin film hydration methods. Liposomes manufactured by both fabrication methods displayed similar cumulative drug release profiles. For Dex-FITC 4k, low encapsulation efficiency was observed throughout for microfluidics, which were attributed to the small size of the drug and the relatively larger pore size of the bilayer, facilitating drug escape. For Dex-FITC 150k, there was a difference in encapsulation efficiency between fabrication methods. Microfluidics fabrication method was superior to thin film hydration. It was hypothesized that drug has more interaction with the lipid during the nanoprecipitation in the microfluidics method. This was in contrast to the thin film hydration, in which the film was first formed before the hydrophilic drug was introduced. The effect of charge played a larger role in microfluidics than thin film hydration, leading to increased encapsulation of the charged lipid/drug pair over the neutral pair. For thin film hydration, most of the drugs were likely to diffuse from the surface of the liposome than the aqueous core, as the amount left in the liposomes were lower than that in microfluidics. The findings in this chapter could lead to

understanding the effect of microfluidic fabrication with drug payloads of larger molecular weight, biomaterials such as peptides, proteins and genetic materials such as siRNA and mRNAs.

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Chapter 7 Discussion and Future Work

In this chapter, the summary of all experiments and hypothesises are provided. In the initial part, a condensed review of microfluidics as a possible fabrication method for liposomes were shown. Various aspects from mechanistics studies to drug encapsulation studies were also drawn together to evaluate its future as a scalable industrial method. In the second part of this chapter, future work and opportunities with the current understanding of microfluidics were discussed.

7.1 Discussion

Developing a therapeutic formulation is daunting and arduous. In the past five decades of research, only a handful of lipid formulations managed to succeed in clinical trials. Liposomes are biocompatible, able to be altered with markers for specificity and prolonged circulation times, and biodegradable. In addition, liposomes can carry both hydrophobic and hydrophilic entities, making it a universal carrier system. For a clinical formulation to proceed from a lab to a final product, there are many considerations. Some of them include the ease of scaling-up, amount of technical expertise required, and the time needed for fabrication/processing. Conventional ethanol injection fabrication method saw its fair share of disadvantages. Microfluidics appears to be a universal, time-saving method with precise size control over liposome formation. There are currently numerous literatures documenting the use of microfluidics for the fabrication of liposomes [1]–[4].

While there is extensive literature showing various liposome systems successfully self-assembling via 2-channel microfluidics, the mechanism behind aggregation and why it occurs in certain lipids, has not been explained before. In the Chapter 5 of the present study, it was demonstrated *for the first time* that the presence of ethanol in microfluidics induces lipid interdigitation leading to size aggregation and instability of microfluidic-formed liposomes, particularly in saturated phosphatidylcholines. It was previously showed that DPPC liposomes undergo interdigitation in microfluidics in the presence of ethanol, and no interdigitation was observed if temperature was maintained above its main transition temperature or ethanol removal was performed above its T_m . The presence of lipid interdigitation was corroborated by the existence of T_m hysteresis of heating and cooling curves in DSC and reduction of d-spacing in SAXS, both characteristic of interdigitation. Furthermore, the inhibiting effect of cholesterol on the membrane interdigitation was studied together with subsequent liposomal formation by microfluidics. The results showed that cholesterol prevents interdigitation even in the presence of ethanol, maintaining its structural integrity.

This finding has important implications for the use of microfluidics fabricating saturated phosphatidylcholines liposomes and offers researchers ways to circumvent aggregation or formulate liposomes that can perhaps take advantage of this phenomenon.

Logically, the next action was to formulate liposomes which were able to escape the phenomenon of interdigitation, while attempting to encapsulate drugs of different hydrophobicity. In the Chapter 6, a hydrophobic model drug Latanoprost, an ester prodrug, for lowering intraocular pressure was used. In this study saturated/unsaturated lipids with and without cholesterol were attempted. It was shown that saturated lipids were not as efficient as unsaturated lipids in encapsulating hydrophobic drug due to lesser hydrophobic regions for the drug to be embedded. By the same logic, lipid formulations that include cholesterol has an overall lower encapsulation efficiency. These findings suggest that EPC was more suitable than saturated lipid counterparts in the encapsulation of Latanoprost. Another area that was explored was the effect of process parameters on the encapsulation efficiency of Latanoprost. While process parameters and fabrication techniques were varied, the encapsulation efficiency was not affected. Not only that, the drug release profile for liposomes fabricated by microfluidics and thin film hydration were alike. Latanoprost, being a hydrophobic drug, had similar partition coefficients in liposomes fabricated using both Microfluidics and Thin film hydration. This suggests that the capacity for loading the drug to be similar, and that is why the results for both methods are congruent.

A next model drug was hydrophilic Dextran-FITC of varying molecular weights and charge. In this chapter, it was important to understand the effect of size of drug, drug/lipid charge and fabrication methods on the encapsulation efficiency of Dex-FITC. Firstly, small hydrophilic drugs had very low loading due to the ease of escape by nature of its small size of the molecule. Results showed that microfluidics did not elevate the situation, they remain easy to escape for Dextran-FITC 4k. Even when there was favourable electrostatic interaction, the encapsulation efficiency was below 10%. When molecular weight of Dex-FITC 150k was used, encapsulation efficiency was observed to be improved for most of the formulations. Among them, it was an interesting finding to see that the neutral drug/lipid pairing had similar encapsulation to the charged drug/lipid pairing. In theory, neutral drug/lipid pairing lack electrostatic interactions with each other, and a lower encapsulation was anticipated. However, it was not the case, and it was suspected that the encapsulation efficiency was overestimated. It was suspected that due to the Dec-FITC 150k being a highly branched structure, could be loosely bounded to the surface of the liposomes during the microfluidic precipitation process. To confirm this suspicion, a drug release on the liposomes was performed. In the first 5 hours, there was statistically more drug released from the neutral drug/lipid pairings and by the 24th hour mark, it was clear neutral drug/lipid pairing had a higher

burst. It was then assumed the remaining drug to be truly encapsulated and recovered the liposomes to verify the true encapsulation. It was clear that in microfluidics, the effect of charge promoted encapsulation of large molecular weight Dextran-FITC.

For the effect of fabrication method on encapsulation efficiency, it was hypothesized that microfluidics was superior to thin film because of the self-assembly mechanism. Figure 7.1 shows the contrast in methods between microfluidics and thin film hydration. In thin film hydration method, because multi lamellar structures was formed before the hydrophilic drugs were introduced, it was likely that the drug had lesser diffusion into the core of the multi lamellar vesicles. Extrusion did not improve the encapsulation as most of the drugs were already in the bulk aqueous medium. Being a passive loading method, there was little driving force to push drugs into the aqueous core of the liposomes. Hence most drug remained on the surface of the liposomes and were quickly observed to be diffusing to the bulk medium, as verified from the drug release curve. True encapsulation efficiencies of both neutral and charged drug/lipid pairings were lower than the microfluidic counterparts. A second centrifugation to remove untrapped drugs also verified this value, that thin film hydration were lower than microfluidics in terms of encapsulation efficiency. These data confirms that microfluidics was more influential than thin film hydration in the encapsulation of large molecular weight Dextran-FITC.

Future studies to formulate liposomes with payloads of various nature built on this knowledge will test this implication.

Figure 7.1: Schematic of liposome fabrication methods. Most drugs in thin film hydration remains in the aqueous medium. Encapsulation improves with the use of microfluidics for large molecular hydrophilic drug Dex-FITC 150k MW.

7.2 Future Work

7.2.1 Exploration of liposomes with a polymeric gel core (Nanolipogel-NLG)

As continuation of this PhD, the exploration of NLG in a microfluidic setup is interesting and an improvement to current liposomal system. NLG contains an aqueous gel core which traps hydrophilic drugs within and slows down release. In the current microfluidic setup, the aqueous phase which is usually PBS, could be substituted with a polymeric solution that cross-links in the presence of UV to form a gel network [5]. Ideally, the lipid encapsulates the polymer within its aqueous core. Excess polymer, photoinitiator, hydrophilic drugs and ethanol could all be removed in one centrifugation step. After removal of excess compounds, the liposomes will undergo cross-linking to form a gel core which traps the hydrophilic drug. In addition, the polymer could be manipulated to have electrostatic interaction with the drug. This could improve encapsulation and slow down drug release. Currently, data shows that small hydrophilic drug escapes out of the liposome bilayer easily, causing either burst release and/or low encapsulation. With the NLG system, it is possible that small hydrophilic drugs can be successfully trapped within the gel core. Some of the polymer properties that could be studied are, polymer molecular weight, cross-link density, amount of polymer used in the fabrication of the NLG. The molecular weight of the polymer determines the mesh size of the gel network, similar to the cross-link density. The amount of polymer used indirectly affects the size of polymer gel as well. Together they affect how much the gel is likely to swell, and whether it will break the liposome structure.

7.2.2 Encapsulation of a commercial drug to evaluate efficacy

Basic mechanistic studies on interdigitation have been completed in this study, as well as hydrophilic and hydrophobic model drugs to evaluate encapsulation efficiency. An increasing important topic to study is mRNA treatment. From the very recent SARS-CoV-2 pandemic, it was clear that mRNA therapeutics would pave the way towards a new era of medical innovation [6]. Microfluidics technology is poised towards this development, because of its benefits mentioned previously. One of the most prominent benefit is the ability for its carrier to bind closely to the genetic material while mixing, increasing its fate in being encapsulated. In addition, organic solvent present within the mixture allows the bilayer structure to be flexible, so that the bilayer can envelop the genetic material. Several studies have begun on using Lipid Nanoparticles (LNP) as a carrier for DNA [7], siRNA [8], and even proteins [9]. More can be done on exploring the different genetic materials, immunogenicity of these carriers, as well as the long-term stability of this system. In vitro studies of cells interaction with the loaded liposomes or LNP could shed light to the efficiency of microfluidic technology both as a stable and scalable way of producing drug adjuvants. Stability of genetic materials remain a huge challenge, and whether the carrier system is able to prevent degradation adequately until it has reached its intended release site. Liposomes serves as a natural and biocompatible carrier system for these types of RNAs. In addition, additives such as cholesterol could be added to further improve the stability of the bilayer, reduce membrane permeability and so prevent degradation by enzymatic reactions. Ultimately, microfluidics serve as a universal platform for the fabrication of these lipid carriers and the ease of scaling ensures its relevance industrially.

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