Establishing and Optimizing a Lipid Nanoparticle Encapsulation Process for Production of Stable mRNA-LNPs

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Abstract

Following their unexpected success during the SARS-COV-2 pandemic, messenger RNA (mRNA) vaccines and therapeutics have altered the vaccination and gene therapy landscapes. Due to the speed and ease of their manufacturing as compared to viral vectored vaccines, mRNA-based vaccines have presented a promising platform technology, especially in the context of pandemic preparedness efforts. However, the entry of mRNA therapeutics into the clinical space hinges on the use of lipid nanoparticles (LNPs). For effective treatment, the mRNA stability must be protected in a cell membrane permeable delivery vehicle, capable of releasing the nucleic acid payload once it reaches the cytoplasm. LNPs, as highlighted by 3 FDA approvals, show great promise in fulfilling these delivery roles. However, despite their advancement into the clinic, there remains key challenges surrounding the manufacturing and thermostability of mRNA-LNPs.

This study aims to establish and optimize microfluidic LNP production parameters as well as improve the stability of mRNA-LNPs through the thorough characterization of the experimental product. We explore the use of widely known and custom microfluidic architectures for rapid mixing and analyze the impact of the type of microfluidic mixer, the total flow rate, and the lipid type on the LNP encapsulation efficiency, size and polydispersity index. Herein, we demonstrate the viability of in-house fabricated microfluidics as a technique for mRNA-LNP production. The results illustrate our ability to produce LNPs within the acceptability criteria (~80% encapsulation efficiency, <200nm in size, PDI <0.3) using several microfluidic architectures. In parallel, we examine the use of excipients that aid in the preservation of these molecules throughout freeze-thaw stresses and long term storage at room temperature, 4, -20 and -80°C. Specifically, the effect of the addition surfactants (P188 and F127) and sugars (Sucrose, Trehalose and Mannitol) were evaluated. Here, we show the ability of select excipients to improve upon the stability of the particles through both freeze-thaw stresses as well long term storage as compared to the control (unformulated samples).

Résumé

Suite à leur succès inattendu lors de la pandémie de SARS-COV-2, les vaccins et les thérapies à base d'ARN messager (ARNm) ont modifié les industries de la vaccination et de la thérapie génique. En raison de la rapidité et de la facilité de fabrication des vaccins à base d'ARNm par rapport aux vaccins aux vecteurs viraux, ils ont constitué une plateforme technologique prometteuse, en particulier pour les efforts de préparation aux pandémies. Notablement, l'entrée des thérapies à base d'ARNm dans l'espace clinique et leur succès dépendent de l'utilisation de nanoparticules lipidiques (NPLs). Pour un traitement efficace, la stabilité de l'ARNm doit être protégée dans un vecteur capable de pénétrer la membrane cellulaire, capable de libérer l'acide nucléique une fois à l'intérieur du cytoplasme. Les NPLs, comme en témoignent les trois approbations de la FDA, sont très prometteuses pour remplir ces rôles de livraison. Cependant, malgré leur avancée en clinique, il reste des défis majeurs concernant la fabrication et la thermostabilité des NPLs à ARNm.

Cette étude vise à établir et à optimiser les paramètres de production microfluidique des NPLs ainsi qu'à améliorer la stabilité des NPLs-ARNm grâce à une caractérisation approfondie du produit expérimental. Nous explorons l'utilisation d'architectures microfluidiques largement connues et personnalisées pour un mélange rapide et analysons l'impact du type de mélangeur microfluidique, du débit total et du type de lipide sur l'efficacité d'encapsulation, la taille et l'indice de polydispersité des NPLs. Nous démontrons la viabilité de nos microfluidiques fabriqués en interne comme technique de production de NPL d'ARNm. Les résultats illustrent notre capacité de production des NPLs en respectant les critères d'acceptabilités (~80% d'efficacité d'encapsulation, <200nm de taille, indice de polydispersité <0.3) en utilisant plusieurs architectures microfluidiques. En parallèle, nous examinons l'utilisation d'excipients qui aident à la préservation de ces molécules à travers le stress de congélation-décongélation et le stockage à long terme à température ambiante, 4, -20 et -80°C. L'effet de l'ajout de surfactants (P188 et F127) et de sucres (saccharose, tréhalose et mannitol) a été évalué. Nous démontrons ici la capacité de certains excipients à améliorer la stabilité des particules à la fois en cas de stress de congélation-décongélation et de stockage à long terme par rapport au contrôle non formulé.

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Contribution of Authors

The present work was written and edited, in its entirety, by myself. This thesis is comprised of a literature review (Chapter 1), and two manuscript based chapters (Chapter 2, 3) which have not yet been submitted. The contribution of all other tentative authors in the work are listed below.

Chapter 1:	**Introduction/ Literature Review**
Contributions:	Prepared and Written Entirely by Maryam Youssef
Chapter 2:	**Streamlining mRNA-LNP Production: Exploring and Characterizing Microfluidic Formulation Techniques**
Authorship:	**Maryam Youssef,** Ayyappasamy Sudalaiyadum Perumal, Cynthia Hitti, Julia Puppin Chaves Fulber, Amine Kamen
Contributions:	**MY:** Project conception, Experimental Plans and Designs, Investigation, Analysis, Writing. ASP: Experimental Plans and Designs, Investigation (Microfluidic Design and Master Wafer Fabrication). CH, JPCF: Investigation (DNA and RNA production for IVT). AK: Supervision, Funding Acquisition
Chapter 3:	**Across Time and Temperature: Evaluating the Stability of mRNA-LNPs through Freeze Thaw Stresses and Long Term Storage**
Authorship:	**Maryam Youssef**, Cynthia Hitti, Julia Puppin Chaves Fulber, Ayyappasamy Sudalaiyadum Perumal, Amine Kamen
Contributions:	**MY:** Project conception, Experimental Plans and Designs, Investigation, Analysis, Writing. CH, JPCF: Experimental Insight, Investigation (DNA and RNA production for IVT). ASP: Experimental Insight. AK: Supervision, Funding Acquisition

Additional research and contributions conducted during this Master's but not included in this thesis have been published in the following works:

- **Youssef M**, Hitti C, Puppin Chaves Fulber J, Kamen AA. Enabling mRNA Therapeutics: Current Landscape and Challenges in Manufacturing. Biomolecules. 2023 Oct 9;13(10):1497. doi: 10.3390/biom13101497. PMID: 37892179; PMCID: PMC10604719.
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List of Acronyms

ARCA	Anti- reverse cap analog
COVID-19	Coronavirus disease 19
CQA	Critical quality attribute
CRISPR CAS-9	Clustered regularly interspaced short palindromic repeats
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DODAP	1,2-dioleoyl-3-dimethylammonium-propane
EE	Encapsulation efficiency
F127	Poloxamer 407
GMP	Good manufacturing practice
IVT	In vitro transcription
LMIC	Low and middle income countries
LNP	Lipid Nanoparticle
mRNA	Messenger ribonucleic acid
NRC	National research council
P188	Poloxamer 188
PAMAMs	Poly(amidoamine) Dendrimers
PAT	Process analytical technologies
PDI	Polydispersity index
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PLGA	Poly-lactic-co-glycolic acid
RNA	Ribonucleic acid
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
siRNA	Small interfering ribonucleic acids
TLR	Toll like receptor
UTR	Untranslated Region

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Chapter 1: Current Landscape and Challenges in the Manufacturing of LNP based mRNA Vaccines and Therapeutics

1 Introduction to mRNA Technology

Recent advances and discoveries in the field of messenger ribonucleic acid (mRNA) therapeutics and vaccinations have enabled the development of scalable, rapid manufacturing processes for these molecules. The technology relies on the transfer and eventual translation of a synthetically encoded antigen or therapeutic protein in the cytoplasm, as is its natural role within mammalian cell biology. This strategy's potential was substantiated after the emergence of SARS-COV-2, due to the vaccines which were developed in record time and were credited in the alleviation of the burden of the pandemic.

The conventionally delivered mRNA is a single stranded, negatively charged molecule, composed of 5 main structural elements: the 5' Cap, 5' and 3' UTRs, the 3' poly A tail and the coding sequence containing the gene of interest [1]. Each structural element plays a critical role in maintaining the stability and improving the translation efficiency of the mRNA molecule. From a manufacturing perspective, this technology provides major advantages over other traditional viral vector and protein subunit vaccines. The molecule is manufactured through a cell-free production process, termed *in-vitro* transcription (IVT), which can be completed within hours or days [2]. The process relies on the sequential enzymatic incorporation of nucleotides following the provided linearized DNA template.

The technology is amenable to the development of a platform manufacturing process, due to its simplicity as compared to protein subunit or viral vectored vaccines. While modification of the open reading frame segment of the mRNA is required to produce a different protein, a large majority of the structural elements of the molecule can remain the same between different products [3]. This allows for the development of a single flexible manufacturing process for several products with a diversity of sequences and lengths. Furthermore, this allows for a streamlined regulatory pathway approach for the approval of mRNA products [4]. Thus, the mRNA technology platform's applications extend far beyond prophylactic vaccination, with many trials currently focusing their efforts on oncological applications [5]. For vaccination and immunotherapeutic applications, the expression of the translated antigen will result in the induction of an immune response, resulting

in the protection against the antigen administered [6]. For protein replacement therapeutics, a critical under-translated or unavailable protein is supplied to the patient through repeated dosing of mRNA encoding the required molecule [7]. Furthermore, this platform can be applied towards cell based therapeutics with many implementing the use of mRNA encoded CRISPR CAS-9 genome editing hardware [8].

2 Challenges in mRNA Stability

Historically, the progression of mRNA vaccines and therapeutics into the clinical landscape has been limited by challenges related to the stability and immunogenicity of the molecule. While the first demonstration of mRNA therapeutic injection in mice dates back to 1990 [9], focus was drawn from in-human trials of mRNA for many years due to their unstable nature. Two main challenges are known to reduce the half-life and prevent the availability of the delivered mRNA sequences into the cytoplasm: (1) Degradation due to extracellular ribonucleases and (2) their inability to pass through the cell membrane. Extracellular ribonucleases, a naturally evolved defence against viruses and exogenous RNA, pose a large obstacle in the delivery of naked mRNA sequences [10]. This degradation has long created difficulties in producing effective mRNA drugs as the final translated protein yield, and thus therapeutic effect, is dependent on the mRNA lifespan [11]. To compensate for these losses and increase the bioavailability of the mRNA, a larger dose would be required, which would in turn risk to instigate an immune response.

Furthermore, once arrived at the cell membrane, the delivered naked mRNA molecule encounters the second challenge of delivery. The negative charge, hydrophilicity, and size of the molecule prevent its passive diffusion through the cell membrane [12]. Instead, naked mRNA has been shown to be taken up through receptor mediated pathways [13], which have generally been considered ineffective for vaccine and therapeutic delivery. Less than 0.0001% of the initially applied molecules are said to reach the cytoplasm after treatment in these cases [14]. These challenges have slowed the progression of this technology throughout the early 2000s despite its potential. Although these difficulties remain, sufficient research has been conducted with the aim of increasing structural stability and efficient intracellular delivery, allowing the implementation of mRNA technology.

3 Enabling mRNA Vaccines and Therapeutics: Improvements in the Stability of mRNA

Cumulative research surrounding improvements to (1) each of the structural elements of mRNA as well as (2) improvements to the delivery strategies have accelerated the transition from bench to bedside of mRNA therapeutics and vaccines in recent years. The following sections review these improvements and identify remaining challenges to facilitate the development of this class of biologics.

3.1 Advances in Improving Inherent mRNA Instability

As described, the inherent instability and susceptibility of mRNA to degradation limited the potency of mRNA therapeutics and vaccines in their early development. Efforts to individually optimize each segment of the structure have been described over the past several decades. First and foremost, chemical modifications to the nucleotides have been developed to modulate the immunostimulatory impact and to improve the translation efficiency of the mRNA. Traditionally, due to their increased length, the delivery of mRNA molecules was difficult and induced an immune response, slowing their clinical progression [15]. Karikó et al.'s discovery of the incorporation of pseudouridine into mRNA demonstrated that the incorporation of modified nucleotides suppresses the activation of the immune response activated through the TLR pathways [16]. Furthermore, the authors observed increased translational ability *in vitro* and *in vivo* of the modified mRNA as compared to the unmodified mRNA [16]. A study conducted by Mauger et al. confirmed that chemically modified nucleotides can enable an increase in stability and expression of mRNA [17]. A myriad of chemical nucleotide modifications has since emerged and been reviewed [18].

To complement these findings, several studies also explored structural modifications to the 5' cap and poly A tail in order to further stabilize the molecule and increase its bioavailability (Figure 1.1) [19]. Notably, many cap analogs, chemically modified variants of the 5' cap, were presented in order stabilize the synthetically transcribed mRNA [20, 21]. For example, anti-reverse cap analogs (ARCA) were easily implemented into the *in-vitro* transcription process and demonstrated improved protein expression than the traditional capped mRNA [22]. Additionally, the 3' and 5' UTRs, which are known to be stabilizing elements of the mRNA have also been targeted for

modification by several researchers [12]. In one instance, Holcik et al. found that modifications in the UTRs increased mRNA half-life from 10h to 30h [23].

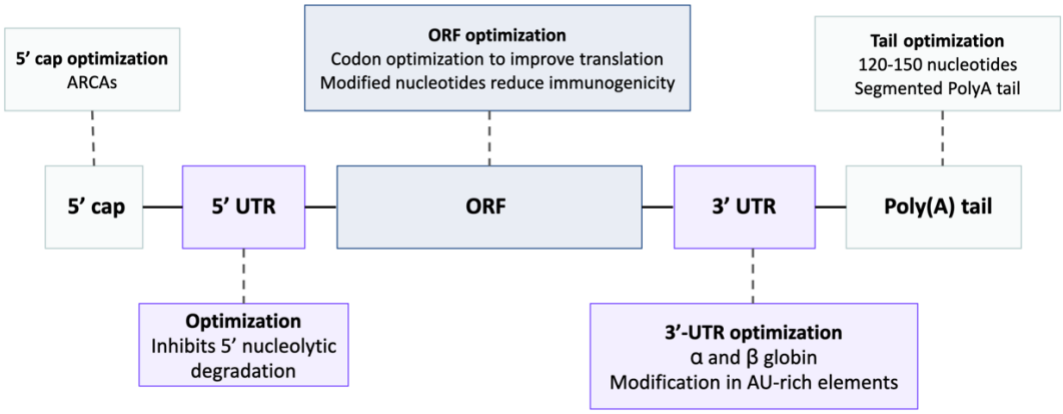

Figure 6.1 Structure and function of five main mRNA elements as well as common modifications made to these elements to increase molecule stability. Figure adapted from [24].

3.2 Advances in Overcoming Intracellular Barriers: Non- Viral Delivery Modalities

As their inherent instability was addressed throughout the early 2000s, effective delivery modalities remained a hurdle to propel IVT-mRNA based biologics into the clinical landscape. Traditionally, viral based approaches have consistently represented promising methods for gene delivery [25]. Viral vectors are evolutionarily equipped with mechanisms which overcome extracellular and intracellular delivery challenges [26]. However, although several research groups have implemented this technology [27-29], these delivery vectors introduce additional manufacturing challenges, cargo size limitations, and immunological concerns [30]. Therefore, many non-viral delivery materials have been adapted for the delivery of therapeutic mRNA. This application benefited from previously ongoing research in the fields of nanotechnology [31], plasmid DNA delivery and siRNA delivery [32]. The materials which have been used can be categorized as illustrated in Figure 1.2. However, the most notable systems which have become of interest for efficient delivery of IVT-mRNA are polymeric and lipid-based delivery systems [33].

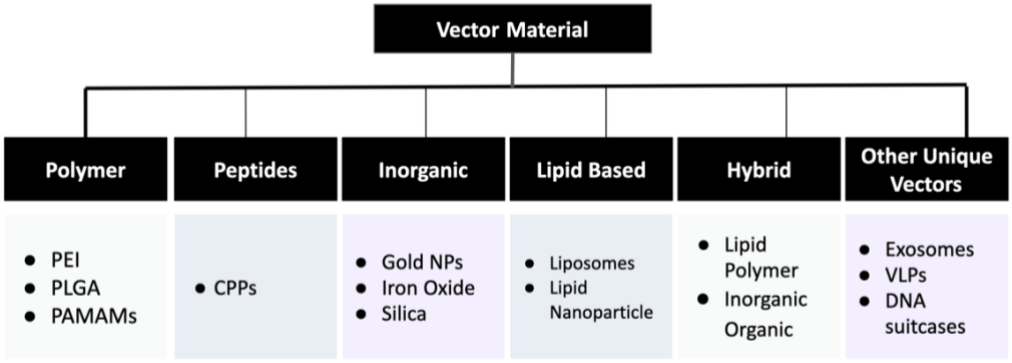

Figure 1.7. Types of Non-Viral Delivery Modalities for IVT- mRNA [34-37].

3.2.1 Polymer Based Systems

The use of cationic polymer based-RNA delivery has been successful in *in-vitro* studies, animal studies, and has recently reached the clinical trial space [38, 39]. The choice of cationic polymers is due to the anionic charge present on RNA molecules, allowing for the complex formation between the two via electrostatic interactions [40]. Polyethyleneimine (PEI), poly(D,L-lactide-co-glycolide) (PLGA), and poly (amido amines) are among the polymers commonly selected for RNA delivery [40].

Polyethyleneimenes have been considered as gold standards for non-viral transfections and are perhaps the most commonly used polymer for gene delivery. The technique was first discovered by Boussif et al. in 1995 for the delivery of DNA into endothelial and embryonic neuronal cells [41]. The positively charged polymer can be used in either its linear or branched states, at different molecular weights, and will interact with the negatively charged nucleotide phosphate groups through its primary amines [42]. However, biocompatibility concerns have been raised depending on the molecular weight and type of PEI. Cationic properties of PEI mediated delivery of nucleic acids have shown toxicological effects, including cell membrane damage as well as apoptotic changes of the cells [43]. Minimization of PEI molecular weight as well as PEGylation of PEI are among the many strategies used to overcome this toxicity [44, 45]. Many other chemical modifications of PEI to render the molecule more biocompatible and reduce its cytotoxicity have been studied at length, as covered in [46]. As such, studies have employed these strategies to develop the delivery of therapeutic mRNA using PEI. For example, Rejman et al. demonstrate

mRNA transfection into several cell types using PEI but observe lower transfection efficiency than other delivery modalities [47]. They attribute this to the strong affinity of PEI to mRNA, limiting their ability to dissociate once translocated into the cytoplasm [47]. Others have explored PEIs ability to deliver mRNA through non-traditional administration routes. Li et al. use cyclodextrin modified PEI for mucosal delivery of mRNA, which is applicable for infectious disease vaccination as well for immunotherapeutics and genetic therapies targeting mucosal tissues. Their study identifies a successful candidate for dendritic cell activation in the lymph nodes through this administration mode [48]. Beyond the preclinical stage, PEI has also reached the clinical trial space as a carrier for DNA immunotherapeutics targeting cancer [49]. As of July 2023, 38 studies have used PEI to deliver genetic therapies in clinical trials, with only 3 of those studies being for RNA delivery [50]. However, to our knowledge, there have been no approved mRNA therapeutics using PEI as a delivery vehicle.

Other polymers have also been explored in literature as alternatives to PEI. PLGA, which presents less biocompatibility challenges, is a neutral molecule commonly used to deliver small molecule drugs. It has been used to deliver mRNA therapeutics through surface modifications, such as the addition of positively charged chitosan to the PLGA [51]. Another approach is the use of poly amido amines (PAMAMs) for gene delivery, first demonstrated in 1993 by Haensler et al. [52]. For example, PAMAM dendrimer formulations have been used to deliver therapeutic mRNA targeting cancer in mouse models, with results that are applicable to the treatment of other genetic disorders [53]. Lastly, the development of "smart polymers", is of interest in the mRNA delivery space. These are polymers which are designed to be stimuli responsive, through changes in their three dimensional structures based on their environment (pH, temperature, etc.) [54]. Yang et al. demonstrate that a pH sensitive polymer complex aided in the stabilization of in vivo mRNA delivery [55]. Despite these advancements, there are currently no approved polymer based mRNA therapeutics or vaccines.

3.2.2 Lipid Based Delivery Systems

Although polymers have received a considerable amount of attention in literature, the most clinically advanced delivery systems for mRNA are lipid based systems. Lipid based delivery systems for nucleic acid delivery were first exemplified in 1987 by Felgner et al., who introduced

the lipofection technique in order to deliver DNA to cells *in-vitro* [56]. This marked the first occasion of non-viral delivery for gene therapy. Following this breakthrough, several reports of lipid based transfection in different cell lines were published, paving the way for the LNP platform which was eventually used to deliver the SARS-COV-2 vaccines (Figure 1.3). However, lipid based delivery systems were discovered and established prior to their application towards gene delivery [57]. In 1965, multilamellar liposomes, which are characterized by their aqueous core and lipid bilayers, were discovered by Bangham et al. [58]. Following this finding, a method to encapsulate drugs and other molecules within the liposomes was demonstrated in the early 1970s, accelerating the drug and gene delivery fields [57]. Liposomes for nucleic acid delivery range between 20 - 1000 nanometers (nm) in size and are generally composed of a cationic lipid along with stabilizers such as cholesterol [59]. Despite their success *in-vitro*, permanently charged liposomes have been unsuccessful in the clinical space due to toxicity, and the use of neutral lipids proved inefficient at encapsulating the negatively charged nucleic acids [60].

In 1997, a work published by Pieter Cullis' group introduced the first ionizable lipid, DODAP [61], altering the liposomal delivery landscape. Ionizable cationic lipids, which acquire a positive charge according to the surrounding acidic pH, and return to neutral charge once at physiological pH, surmounted the toxicity issues associated with traditional liposomes and led to the creation of ionizable LNPs [62]. Their positive charge at low pH allowed efficient interaction and complex formation with the nucleic acids, while reducing concerns associated with cationic liposomes.

Lipid nanoparticles are characterized by a solid core, several lipid layers and microdomains containing the oligonucleotide [63]. LNP formulations include four distinct categories of components: (1) an ionizable cationic lipid, (2) a PEGylated lipid, (3) cholesterol, and (4) a phospholipid [64]. The ionizable lipid, plays a critical role not only in the encapsulation of the nucleic acid but also in its release. As the LNP is uptaken through an endosomal pathway, the ionizable lipid will regain its positive charge through the acidic environment present within endosomes [60, 65]. The newly positively charged ionizable lipid interacts with the negatively charged lipids present on the inner layer of the endosome, destabilizing the membrane, and inducing the release of the mRNA cargo [66]. The PEG-lipid conjugate is necessary to maintain LNP stability, reduce aggregation between particles and to increase the circulation half-life of the

particles [64]. The cholesterol is included as a medium to maintain membrane rigidity, but also facilitates the encapsulation of the nucleic acid[64]. Lastly the phospholipid's primary role is to facilitate intracellular delivery[64].

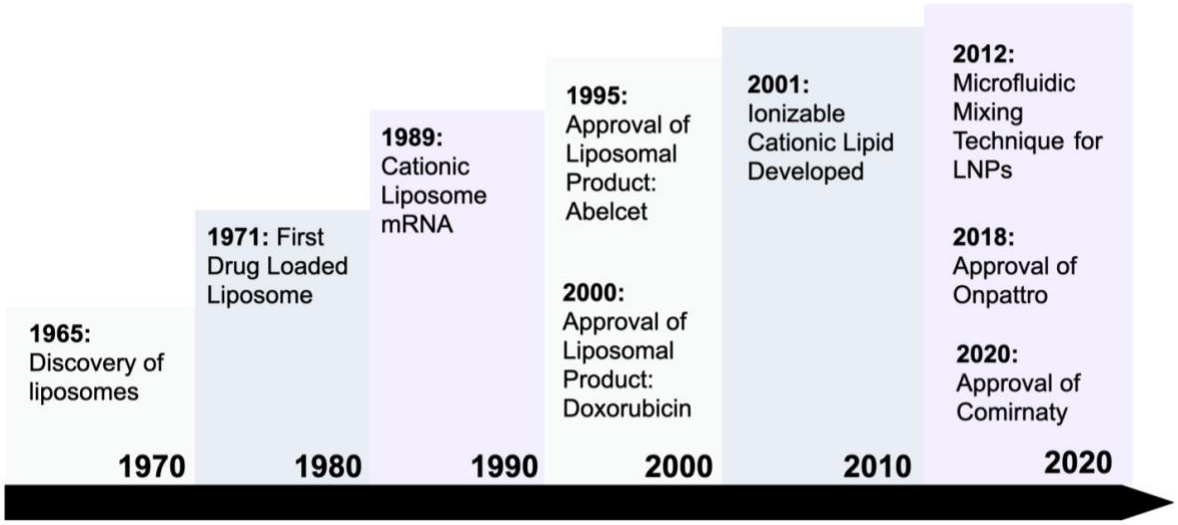

Figure 1.8. Timeline of the Milestones leading to the development of Lipid Nanoparticles for the delivery of IVT- mRNA. Figure adapted from [57].

4 Success of Lipid Nanoparticles: Emergence of mRNA-LNPs and Ongoing Work

In 2018, FDA approval of Onpattro, an ionizable LNP based siRNA drug, marked the first regulatory approval of an LNP-RNA drug, opening up a realm of possibilities for the development of mRNA therapeutics and vaccines [67]. This paved the way for the approval of the BNT162b2 and mRNA-1273 vaccines during the COVID-19 pandemic.

The lipid component molar ratios, the total lipid concentration, and lipid to mRNA ratios have all been varied across approved LNP products and across the published literature. Roces et al. have demonstrated that adjusting these parameters impacts the physicochemical characteristics of the particles, including the size and zeta potential [68]. In addition to the manufacturing method, these parameters can impact the RNA encapsulation efficiency, the particle size, the polydispersity index (PDI) and the potency of the product [69]. Previous work, such as that of Sago et al., has included the production of LNP libraries (>250 mRNA-LNPs) by varying the ionizable lipid used and the lipid molar ratios in order to determine which LNP performed best in *in vivo* delivery [70]. Their work demonstrated that optimization of the composition of the LNP impacts delivery efficiency and tissue tropism. Furthermore, Kauffman et al. emphasize these findings by demonstrating that

the simultaneous optimization of the mRNA: lipid weight ratio as well as the lipid structures included in the LNP through a design of experiment approach led to a significant increase in protein expression [69]. Interestingly, despite these findings, all approved LNP products have similar disclosed lipid molar ratios (Table 1.1) [71]. For the purposes of the forthcoming work, a ratio of 50:10:38.5:1.5 (Ionizable lipid: Phospholipid: Cholesterol: PEG-lipid) is used due to its clinical applicability. Similarly, ALC-0315 and SM-102 are used in our studies due to their clinical relevance.

Product Name	Lipid Ratio	Ionizable Lipid	Phospholipid	PEG-lipid
Onpattro	50:10:38.5:1.5	DLin-MC3-DMA	1,2-DSPC	ALC-0159
BNT162b2	46.3:9.4:42.7:1.6	ALC-0315	1,2-DSPC	PEG2000-DMG
mRNA-1273	50:10:38.5:1.5	SM-102	1,2-DSPC	PEG2000-DMG

Table 5.1. LNP composition of approved LNP-RNA products.

In addition to the ongoing work to optimize and introduce new lipid compositions for lipid nanoparticles, a large portion of the field is currently contributing to the development of methods for the targeting of these particles. It is known that the administration of LNPs results in the non-specific accumulation of the particles in the liver and are eventually eliminated [72]. Specifically, this phenomenon is observed when administration of the particles is through intravenous and intramuscular routes [73]. In order to facilitate their clinical translation, overcoming this accumulation with the ability to induce tissue specific uptake is imperative. Currently, the accumulation of these LNPs risks to induce or exacerbate pre-existing inflammation in the patient after administration [74, 75]. To this end, protocols such as that for the preparation of the selective organ targeting (SORT) nanoparticles, have enabled the ability to alter tissue tropism [76]. In this work, Cheng et al. describe the use of different percentages of DOTAP, a permanently charged cationic lipid, to redirect the particles in in-vivo models [76]. This method allowed them to alter the expression profile from the liver to the spleen or lung. Others have shown the successful targeting of dendritic cells through modifications in the RNA to lipid ratio [77]. Other strategies, including the conjugation of antibodies to the surface of LNPs have been considered, specifically for oncological applications [78]. These efforts to control the biodistribution of the particles are anticipated to reduce the burden for large dosage manufacturing if a more potent response, with less off-target effects, is achieved through a targeted approach.

4.1 Clinical Applications

LNPs provide a versatile platform for delivery, amenable to many applications. This has allowed these particles to become widely used in the clinical space, beyond the context of vaccinations and pandemic use [79]. In a previously published work, we summarized the currently ongoing (not yet recruiting, recruiting, active) lipid- based mRNA therapeutics trials [80], and have updated the table as shown below to include all ongoing mRNA- LNP therapeutic trials as of May 2024 (Table 1.2).

Trial ID	Status	Indication	Treatment Name	Dose Regimen	Administration Method
NCT06389591	Not yet recruiting	Recurrent Glioblastoma	pp65 RNA loaded lipid particles, pp65 RNA-LPs/ RNA loaded lipid particles	N/A	IV
NCT06243770	Recruiting	Healthy Individuals	mRNA-0184	4 doses over 16 weeks	IV
NCT06088004	Recruiting	Solid Tumor, Adult	ABO2011 Injection	N/A	IT
NCT06147856	Recruiting	Phenylketonuria	mRNA-3210	Every 1-3 weeks. 12 doses total	IV
NCT06249048	Not yet recruiting	Advanced Solid Tumor	STX-001	N/A	IT
NCT04573140	Recruiting	Adult glioblastoma	Autologous total tumor mRNA and pp65 full length (fl) lysosomal associated membrane protein (LAMP) mRNA loaded DOTAP liposome	Every 2 weeks (3 cycles), Monthly (15 cyles)	IV
NCT05097911	Recruiting	Advanced Hepatocellular Carcinoma	MTL-CEBPA	Day 1 & Day 8 of a 21 Day Dosing Schedule	IV
NCT05579275	Recruiting	Advanced malignant solid tumors	JCXH-212 Injection	Every 3 weeks (up to 8 cycles)	Unspecified injection
NCT05949775	Not yet recruiting	Advanced Malignant Solid Tumours	Neoantigen mRNA Personalised Cancer vaccine	Every 3 weeks (9 cycles)	SQ
NCT05978102	Recruiting	Advanced Solid Tumor	STI-7349 / IL2v mRNA	Every 3 weeks	IV
NCT05533697	Recruiting	Advanced Solid Tumours	mRNA- 4359	N/A	IM
NCT02872025	Recruiting	Carcinoma, Intraductal, Noninfiltrating	mRNA 2752	2-4 Doses	ILES

NCT05659264	Recruiting	Chronic heart failure	mRNA-0184	2 groups: single dose OR 4 doses every 16 weeks	IV
NCT05141721	Active, not recruiting	Colorectal neoplasms	GRT-R902 (samRNA), GRT-C901(viral vector)	4 Doses over first year	IM
NCT05712538	Recruiting	Cystic Fibrosis	ARCT-032	Single dose	INH
NCT05668741	Recruiting	Cystic Fibrosis	VX-522	Single dose	INH
NCT05938387	Active not recruiting	Glioblastoma	CV09050101 mRNA vaccine	7 Doses at different intervals	IM
NCT05095727	Recruiting	Glycogen storage disease	mRNA-3745	Single Dose. Additional dosages after > 21 days	IV
NCT05497453	Recruiting	Hepatocellular Carcinoma	OTX-2002	At least 2 Doses	IV
NCT04710641	Active, not recruiting	Hepatocellular Carcinoma	MTL-CEBPA (saRNA)	Every 3 weeks	IV
NCT05120830	Active, not recruiting	Hereditary Angioedema	NTLA-2002	Single Dose	IV
NCT05933577	Recruiting	High Risk Melanomaa	V940	Every 3 weeks (up to 9 doses)	IM
NCT05295433	Recruiting	Isolated methylmalonic acidemia (MMA)	mRNA-3705	Every 2-4 weeks	IV
NCT04899310	Recruiting	Isolated methylmalonic acidemia (MMA)	mRNA-3705	Every 2-4 weeks	IV
NCT03289962	Active, not recruiting	Locally or Advanced Metastatic Cancer	RO7198457	Every 2 Weeks	IV
NCT05969041	Recruiting	Malignant Epithelial Tumours	MT-302 (A)	Weekly - Biweekly doses for first 3 doses. Every 4 weeks for upcoming	IV
NCT05539157	Active not recruiting	Malignant solid tumours, etc.	JCXH-211	Every 4 weeks (Up to 3 doses)	IT
NCT05714748	Recruiting	Malignant Tumours	EBV mRNA vaccine	Weekly (4 doses), Followed by 1 month interval (1 dose)	IM
NCT03897881	Recruiting	Melanoma	mRNA-4157	Every 3 weeks. Up to 9 Doses.	IM

NCT04526899	Active, not recruiting	Melanoma	BNT111	N/A	IV
NCT05142189	Recruiting	Non-Small Cell Lung Cancer	BNT116	N/A	IV
NCT04442347	Active not recruiting	Ornithine Transcarbamylase Deficiency	ARCT-810	Single Dose	IV
NCT05526066	Recruiting	Ornithine Transcarbamylase Deficiency	ARCT-810	Every 2 weeks (Up to 6 doses)	IV
NCT04161755	Active, not recruiting	Pancreatic cancer	RO7198457	Every Week (8 Cycles)	IV
NCT05130437	Recruiting	Propionic Acidemia	mRNA-3927	Every 3 weeks	IV
NCT04159103	Recruiting	Propionic Acidemia	mRNA-3927	Every 3 weeks. Up to 10 Doses.	IV
NCT05660408	Not yet recruiting	Pulmonary osteosarcoma	RNA-LP vaccine	Every 2 weeks (2 Cycles), Monthly (12 cyles)	N/A
NCT03739931	Active, not recruiting	Relapsed solid tumor malignancies/ lymphoma	mRNA-2752	Every 2 Weeks	IT
NCT04503278	Recruiting	Solid Tumor	BNT211- CLDN6 CAR-T/CLDN6 CAR-T(A), CLDN6 RNA-LPX	N/A	IV
NCT05262530	Recruiting	Solid Tumor	BNT142	N/A	IV
NCT04710043	Recruiting	Solid Tumor	BNT152/ BNT153	N/A	IV
NCT04455620	Active not recruiting	Solid Tumor	BNT151	N/A	IV
NCT03313778	Recruiting	Solid tumours	mRNA-4157	9 cycles (once every 3 weeks)	IM
NCT04601051	Active, not recruiting	Transthyretin-Related (ATTR) Familial Amyloid Polyneuropathy	NTLA-2001	Single Dose	IV
NCT04534205	Recruiting	Unresectable Head and Neck Squamous Cell Carcinoma	BNT113	N/A	IV

Table 1.6. Updated ongoing lipid-based mRNA therapeutic clinical trials as of May 2024.

Table 1.2 demonstrates the need for chronic dosing of these therapeutics. As opposed to prophylactic vaccinations, which are not required to be delivered continuously, a majority of these therapeutics require several cycles of drug delivery. These upcoming mRNA-LNP products highlight the need for an increased understanding of LNP manufacturing methods and thorough characterization of mRNA-LNP products to maintain consistently high levels of protein production

and low immune responses with each dose delivered [79]. Furthermore, we observe that the dose requirement fluctuates significantly across therapeutics. It was noted that those administered through IV tend to be assessed at doses in the milligram dosage range, whereas locally administered mRNA-LNPs are assessed at doses in the microgram dosage range [80]. Furthermore, many mRNA-LNP products have personalized medicine applications [24], meaning that each production batch would be to fulfill only a single patient's dose requirement. This range of dosage requirements illustrates the need for well-defined flexible manufacturing methods for LNPs which are capable of fulfilling scale requirements as small as locally delivered personalized medicine applications and as substantial as vaccination products. The work described hereafter aims to fulfill both the need for a flexible manufacturing platform as well as for characterization of mRNA-LNPs.

4.1.1 Manufacturing Technologies, Characterization and Remaining Challenges

Since the inception of ionizable LNPs, several manufacturing methods and technologies have been developed. Nucleic acid loading of the ionizable LNPs using an ethanol based buffer was first described by Semple et al. in 2001 [62]. In this initial work, they describe an extrusion based method for the production of 80-140 nm particles [62]. As the field has grown, the development of two main LNP preparation strategies have been popularized: (1) T-junction mixing/Turbulent jet mixing and (2) Microfluidic mixing [81, 82]. In each of these cases, the encapsulation of the nucleic acid within the LNP is performed through rapid mixing of the ethanol lipid-containing phase and the aqueous nucleic acid-containing phase.

The T-junction method, which consists of the use of a propylene T-tube and of peristaltic pumps to initiate the mixing between both streams has been used in very few publications at lab scale due to the volume required [82]. The method was introduced in 1999 for the encapsulation of plasmid DNA [83], and further elaborated on in 2005 by Jeffs et al. [84]. In one instance, Goswami et al. applied the technique to encapsulation of self-amplifying RNA and achieved encapsulation efficiencies between ~20-88% depending on the condition [85]. In another, Chen et al. employ the use of T-junction mixing for mRNA-LNP formation, but the critical quality attributes of the prepared particles are not discussed in detail [86]. Following this "macrofluidic" logic, turbulent jet mixers were developed as a method for the encapsulation of nucleic acids into LNPs at mass

production rates. Classes of these mixers, including confined impinging jet mixers, coaxial jet mixers and multiple inlet vortex mixers were introduced into the manufacturing space as potential production methods for LNPs [87]. Lim et al. demonstrated the use of high throughput coaxial jet mixing for the formation of lipid vesicles at flow rates >200 mL/min [88]. Furthermore, this technology was chosen by Pfizer/BioNTech for the preparation of their vaccine during the SARS-COV-2 pandemic. The company employed the use of confined impinging jet mixers which operated continuously to fulfill the dose requirements [89]. These particles were approximately 80nm in size, but their encapsulation efficiency was undisclosed [90]. Despite their success, these technologies are limited by the volumes required for each experimental run (>20mL material) [81]. These volumes are not amenable to exploratory and screening studies but are more appropriate for industrial scale applications.

Among these manufacturing methods, microfluidic mixing devices have presented promising versatile avenues for the production of mRNA-LNPs. Microfluidic devices accommodate small volumes required at lab scale for developmental studies [91], while simultaneously having the capability of being scaled up in terms of volumetric flow or scaled-out in parallel operations [92]. These systems permit the tunable and reproducible mixing of volumes from the nanoliter to liter scale. The method was first introduced in 2012 for the application of siRNA and drug entrapment within lipid nanoparticles by Belliveau et al. and Zighaltsev et al. [93, 94]. Belliveau et al. reported encapsulation efficiencies ranging between 65-95% and the ability to produce LNPs as small as 20nm with little variability [94]. In 2015, this work was expanded upon to include the encapsulation of the larger molecule, mRNA. Leung et al. achieved encapsulation efficiencies of approximately 90% in their demonstration that microfluidic devices were viable for mRNA therapeutic and vaccine manufacturing [95]. Thus, these works solidified the groundwork to establish microfluidics as a flexible reproducible manufacturing platform for mRNA-LNPs. These milestones allowed for Cansino Biologics to utilize this methodology throughout the COVID-19 pandemic for the production of their LNPs using the Precision Nanosystem GMP microfluidic system [96].

Several microfluidic architectures exist, including baffled mixers, ring micromixers, and staggered herringbone mixers, and have been reviewed thoroughly in several publications [97, 98]. To our

knowledge, however, no systematic comparison of these architectures exists for the production of mRNA-LNPs within one body of work. We aim to fill this gap within the upcoming studies described. Furthermore, automated benchtop microfluidic mixers have been commercialized but are expensive equipment to acquire [99, 100], limiting their accessibility. The characterization of cost-effective in-house manufactured microfluidics, which are easily replicated, renders this methodology attainable, and accelerates the screening process and thus the translation of mRNA medicines into the clinical space. It is worth noting, however, that though the above described methods dominate the mRNA-LNP manufacturing space, publications have explored the use of other encapsulation strategies such as reverse phase evaporation and ethanol injection [101].

To qualify each of the above described manufacturing methods, the size, polydispersity index and encapsulation efficiency of the produced particles must be analyzed as described in Table 1.3. General proposed guidelines for the acceptability criteria have been provided but due to the accelerated timeline of the approval of mRNA-LNP products, these guidelines remain unclear and may vary by application.

Critical Quality Attribute	**Analytical Technique**	**Acceptability Criteria**
Size (nm)	Dynamic Light Scattering	<200 nm
Polydispersity Index (PDI)	Dynamic Light Scattering	<0.3
Encapsulation Efficiency	Ribogreen Assay	≥80%
mRNA integrity (%)	Capillary Gel Electrophoresis	>70%

Table 1.7. Process Analytical Technologies (PATs) Required Throughout mRNA-LNP manufacturing [102, 103].

Furthermore, assays to determine the potency of the product vary greatly across literature. Paunovska et al. have previously demonstrated after screening 281 LNPs that in vitro delivery of LNPs is not predictive of their in vivo delivery [104]. Furthermore, Escalona-Rayo et al. observed the same effect when analyzing the currently approved LNP formulations in a mouse model and revealed that the *in-vitro* performance of the particles was not indicative of their *in-vivo* performance [105]. Therefore, there exists a need for the development of *in-vitro* assays which more closely resemble *in-vivo* experiments in order to properly assess the efficacy of the mRNA-LNP product [106]. As of now, a focus is placed on the analysis and characterization of the

physico-chemical characteristics of the mRNA-LNP products and a push for *in-vivo* studies rather than non-predictive *in-vitro* studies.

The aforementioned characterizations and PATs are of increasing importance to assess and improve the current thermal stability of mRNA-LNP products. As mRNA vaccines were popularized during 2020, their instability and required storage conditions became a primary challenge for their distribution [107]. This issue is not limited to the mRNA-LNP vaccine products, however, and continues to place a critical limitation on the various mRNA therapeutics in development, representing a bottleneck in the progression of mRNA technology [108]. Table 1.4 summarizes the primary mechanisms of degradation for mRNA-LNPs, revealing the complexity of addressing all of these pathways.

Mechanism of Degradation	Details	Monitoring Method
Physical Degradation of LNPs	Aggregation of LNPs Fusion of LNPs Leakage of mRNA cargo	DLS Ribogreen Assay
Chemical Degradation of lipid	Hydrolysis, Oxidation of Lipids	HPLC [109]
Hydrolysis of mRNA backbone	mRNA degradation	Capillary Electrophoresis
Oxidation of mRNA nucleobases	mRNA adduct formation	HPLC

Table 1.8 Known mRNA-LNP degradation mechanisms and analytical techniques to monitor them as described in [71, 108, 110].

Despite these difficulties, studies have undertaken the aim of extending the shelf life and easing the ultra-cold storage requirement of mRNA-LNPs since the SARS-COV-2 pandemic. One approach to do so involves the design of novel ionizable lipids to allow for stabilized particles [89, 111-114]. Others have demonstrated the impact of existing lipid compositions on the stability of mRNA-LNPs in non-frozen conditions [115]. Another approach to reduce degradation in the product over storage durations is the addition of excipients and cryopreservatives to protect the product throughout the freezing process. Kafetzis et al. have evaluated the use of sugars such as sucrose and trehalose in the long term storage of mRNA-LNPs and found that sucrose was an effective cryopreservative at -80°C but did not evaluate stability beyond one month of storage

[116]. Similarly, Zhao et al. found that sucrose or trehalose were effective in maintaining mRNA integrity after 3 months of storage, when stored under liquid nitrogen [117]. An earlier work published in 2016 by RL et al. demonstrated the long term aqueous storage of siRNA-LNPs but did not include analysis of mRNA loaded LNPs [118]. Furthermore, in this work, they highlight the changes in size and polydispersity index incurred when freezing and thawing LNPs [118]. These concerns were echoed by Curevac representatives, who demonstrated that as we decrease mRNA-LNP concentrations, these effects become more pronounced [110]. Additionally, researchers have demonstrated the importance of the buffer and its pH in the longevity of mRNA-LNPs. Specifically, Henderson et al. demonstrate that Tris and Hepes buffer outperform PBS in the preservation of mRNA-LNPs at -20°C [119]. Lastly, many have focused their research efforts to provide a solid, lyophilized presentation of mRNA-LNP vaccines and therapeutics, to avoid degradation methods experienced in the presence of water [120-124]. Overall, it is clear that there remains room for improvements to be made to provide improved storage conditions in aqueous and frozen conditions.

5 Summary and Research Aims

In conclusion, chapter 1 reviews the emergence of mRNA-LNPs and establishes their importance within the clinical landscape. However, this chapter also highlights the challenges that have been associated with the manufacturing methods and characterization of these LNPs. It is apparent that there exists a need for affordable manufacturing methods which are capable of serving both the small scale exploratory or personalized medicine needs as well as the large scale production needs currently present within the field as well as analytical strategies to monitor these products. Furthermore, enriching knowledge on the stability profiles of mRNA-LNPs and the proposition of excipients and formulations to improve the current shelf life of these products is critical to ensuring the accessibility and durability of these products. The following thesis aims to address these challenges through the following aims: (1) optimize lipid nanoparticle composition and production process parameters using affordable microfluidic devices and (2) explore formulations for long term stability of mRNA LNPs.

Chapter 2: Streamlining mRNA-LNP Production: Exploring and Characterizing Microfluidic Formulation Techniques

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1 Introduction

Recent approval of the mRNA vaccines for SARS-COV-2, as well as advancements in the rapid manufacturing of mRNA, have presented great promise for the development of other mRNA-based therapeutics and vaccines. Despite their success, messenger RNA molecules are highly susceptible to rapid degradation by ribonucleases and have limited ability to permeate the cell membrane due to their negative charge and hydrophobicity [1]. To facilitate their *in vivo* delivery and their acceleration in the clinical space, an efficient delivery material is required. A growing number of mRNA therapeutics have entered the preclinical and clinical stages, the majority employing the use of a lipid nanoparticle (LNP) as the carrier [2].

In 2018, the approval of Onpattro, an siRNA-based drug, marked the first use of the solid lipid nanoparticle. The particles are composed of an ionizable cationic lipid, a phospholipid, cholesterol and a PEG-lipid. When formed, the lipid nanoparticle contains microdomains encapsulating the therapeutic nucleic acid molecules, and a solid lipid core with a bilayer membrane. The encapsulation efficiency, size and polydispersity index of the particles must be tightly controlled and assessed for mRNA-LNP drug products. Acceptance criteria for each of these attributes have been suggested to ensure the quality of the LNPs. Increasing the encapsulation efficiency (EE%) of the particles is critical to maximize the delivered dose and to reduce the immunogenic response caused by naked RNA and empty LNPs [3]. Thus, publications have indicated that it is preferable that the encapsulation efficiency is approximately 80% or above. Furthermore, it has been demonstrated that particle size can impact the uptake and subsequent immunogenic response, and thus the desired size may vary by application. However, it is suggested that the particles produced should not exceed 200nm in size. Lastly, to ensure a homogeneous population of particles, it is recommended that the polydispersity index (PDI) remains below 0.3 [3].

The production of mRNA-LNPs is commonly performed through rapid mixing of an organic solvent phase, containing the lipids, and an aqueous phase, containing the nucleic acids intended for delivery. Several methods for the preparation of LNPs have been demonstrated, namely, pipette mixing, turbulent jet mixing and microfluidic mixing. Pipette mixing strategies have been used for exploratory studies, but lack scalability, reproducibility and experimental control [4]. Turbulent jet mixing involves the rapid collision of the aqueous and organic solvent streams originating from two jets in opposing directions to form the LNPs. This technology has previously been employed in commercial applications, including during the manufacturing of the BNT162b2 vaccine [5]. However, this method is more suitable for industrial applications due to the limitation of large volume requirements for each experiment [6], limiting the ability for bench to bedside development. The emergence of continuous flow microfluidic mixing for LNP preparation provides a controlled, reproducible strategy with the potential for parallelization and large-scale manufacturing. Microfluidic mixing occurs through the diffusion between the laminar flow between two streams, as well as through the introduction of micromixer structures to induce turbulence in the flow. These cost effective and robust devices facilitate the transition between the screening and the large-scale production stages. Several well-known microfluidic geometries for passive mixing have been used for the preparation of mRNA-LNPs, each uniquely impacting the final quality characteristics of the LNPs [7]. Specifically, T-junction mixers, baffle micromixers and bifurcating micromixers have gained popularity in the manufacturing of RNA-LNPs, with the majority of publications and automated benchtop microfluidic mixers employing the use of baffle and bifurcating mixers.

This study aims to demonstrate the viability of in- house microfluidic devices for the production of mRNA-LNPs. Further, we systematically characterize and compare LNPs produced by widely known and discussed microfluidics to provide data on the cumulative conditions that allow for efficient, scalable mRNA- LNP production. A selection of two dimensional microfluidic geometries within known mixer architectures (serpentine baffle micromixers and bifurcating ring micromixers) were designed (Figure 2.1), fabricated, and operated to produce mRNA LNPs for analysis. Two dimensional microfluidics are chosen due to their ease of fabrication as opposed to three dimensional designs, in order to ensure the accessibility of the presented devices [8, 9]. We evaluate the encapsulation efficiency, size and polydispersity index of the LNPs produced by a

variety of microfluidic architectures. In addition, we evaluate the impact of modifications in the lengths of microfluidic architectures on the final quality attributes of the LNPs.

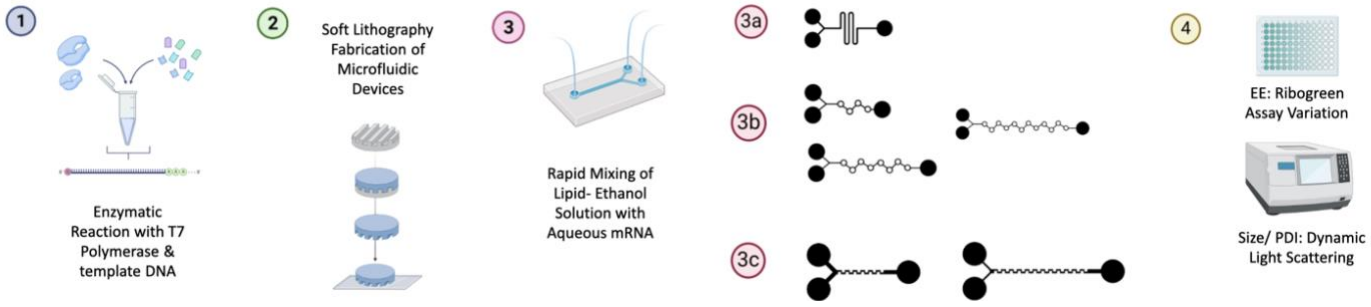

Figure 2.1. Overall Pipeline for Production and Characterization of mRNA-LNPs for the study. (3a), (3b) and (3c) illustrate the evaluated microfluidic device designs for the encapsulation of mRNA into LNPs.

2 Methods and Materials

2.1 mRNA Synthesis and Purification

A DNA construct encoding for eGFP (pGEM4Z-EGFP) was purchased from Addgene and a construct encoding for nLuciferase (pcDNA-LUC-CBR2opt-T7AG-C1) was gifted by the National Research Council of Canada (NRC). *In-vitro* transcription of the two DNA constructs was conducted using the MEGAscript T7 Transcription Kit (Invitrogen) following the manufacturer's protocol. The resulting mRNA was purified by phenol-chloroform purification and quantified using the Quant-it Ribogreen RNA Assay Kit (Invitrogen). RNA sequence length and purity was verified by formaldehyde gel electrophoresis.

2.2 Microfluidic Device Preparation

Microfluidic devices were designed using Fusion 360, and the polarity was applied in AutoCAD. The designs were sent to ArtNet Pro for photomask printing. SU-8 photoliography was used to fabricate the microfluidic features on a silicon wafer. Features of channel heights were 40 µm in height. Copies of the devices were fabricated by soft lithography using polydimethylsiloxane (PDMS SYLGARD 184 silicone elastomer, Dow Consumer Solutions), diced, inlet and outlet ports were punched, and the devices were bonded to a glass surface using a UV plasma treatment

(UV/Ozone ProCleaner Plus, BIOFORCE nanosciences) and heating at 65°C. Mixing in channels was observed under IX83 Olympus confocal microscope, with Rhodamine Green Dye.

2.3 mRNA LNP Formulation & Microfluidic Device Operation

LNPs were formulated through rapid mixing of the ethanol and aqueous phases in each of the microfluidic devices. For the ethanol phase, lipids were resuspended in 100% ethanol at a molar ratio of 50: 10: 38.5: 1.5 (ALC-0315: 1,2-DSPC: Cholesterol: ALC-0159) to a total of 1 mg/mL. ALC-0315 and ALC-0159 were acquired from Cayman Chemical, Cholesterol and 1,2-Distearoyl-sn-glycero-3-phosphocholine were acquired from Sigma Aldrich. For the aqueous phase, the mRNA was diluted in 50 mM sodium acetate (pH 5.0). The lipid to mRNA weight ratio was maintained at 10:1 for all experiments.

Prior to formulation, sealed devices were flushed with RNase Zap (ThermoFisher), followed by ethanol. Using a dual flow rate syringe pump, lipids and RNA were infused at a flow rate ratio of 3:1 (aqueous phase: ethanol phase) for all devices. LNP samples were collected, diluted 20x in PBS, centrifuged at 2000g in 100kDa Amicon Ultra 4 until they reached their initial volumes and stored at 4°C for characterization. Prior to characterization, mRNA LNPs were sterile filtered using a 0.22 μm 4 mm Millex Syringe Filter (Millipore Sigma).

2.4 mRNA LNP Characterization

RNA encapsulation efficiency (EE%) of the LNPs was determined by a modified Quant-it Ribogreen RNA Assay Kit (Invitrogen) protocol. Duplicate LNP samples were diluted 1:50 in either 1X TE buffer, to determine the unencapsulated RNA concentration, or in 2% Triton X-100, to determine the total RNA concentration, in a 96 well plate. A standard curve was prepared using the Ribosomal RNA standard provided in the kit. The plate was incubated for 10 min at 37°C to disrupt LNPs prior to the addition of Ribogreen Reagent into each well and the fluorescence intensity values were measured by the Agilent BioTek Synergy HTX MultiMode Microplate Reader. EE% was calculated as the difference between the total RNA concentration and the unencapsulated RNA concentration divided by the total RNA concentration.

Size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using the ZetaSizer Nano S90 (Malvern Panalytical). LNPs were brought to room temperature and diluted 1:15 in PBS in a 50 µL disposable cuvette (SARTEDT). The material refractive index (RI) was set to 1.37, the dispersant RI was set to 1.34 for PBS and the temperature was maintained at 25°C. Each sample was subjected to 2 runs of at least 10 measurements each.

3 Results

3.1 Impact of Ionizable Lipid on Microfluidic Mixing Performance

Initially, to evaluate the impact of the chosen ionizable lipid on mRNA- LNP mixing efficiency, a classical serpentine channel was used to perform experiments. Both the ALC-0315 ionizable lipid as well as the SM-102 ionizable lipid were evaluated due to their FDA approvals and clinical relevance. We evaluated flow rates varying from 0.04mL/min to >1mL/min for both lipids. Firstly, we confirm that mixing within a serpentine channel is sufficient to produce mRNA-LNPs (Figure 2.2). Results demonstrated that the choice of ionizable lipid impacts the encapsulation efficiency achieved within these devices (Figure 2.2B, 2.2C). In this case, SM-102 consistently outperformed ALC-0315 in its ability to complex with the mRNA and encapsulate it. Specifically, we note that at lower flow rates, the difference between the SM-102 based LNPs and the ALC-0314 based LNPs is more prominent than at higher flow rates, indicating that SM-102 shows a lack of flow rate dependency in its interaction with mRNA within the given flow rates. These results emphasize the importance of the development of novel ionizable lipids alongside manufacturing methods and devices.

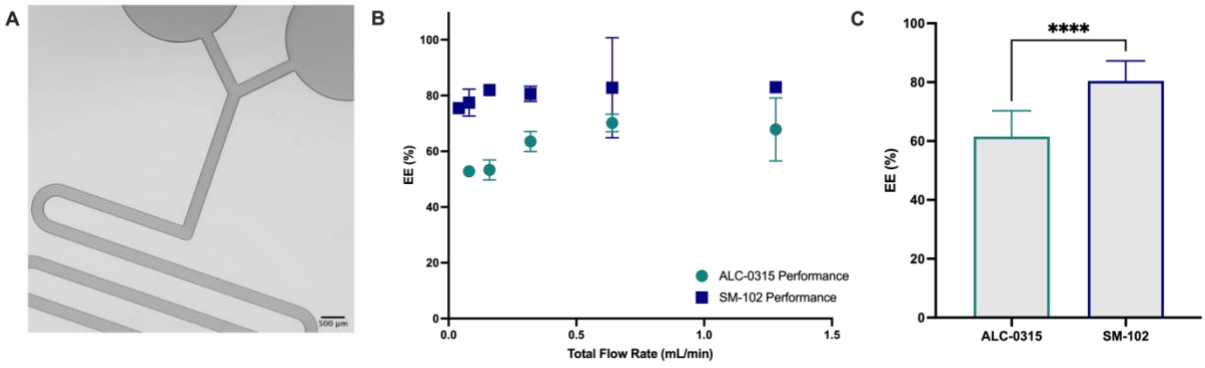

Figure 2.2. Demonstration of the Impact of Ionizable Lipid Choice on mRNA-LNP complexation in (A) microfluidic serpentine channels. (B) Results of encapsulation efficiency as a function of flow rate reveal

the significant differences in the (C) mean encapsulation efficiency achieved with ALC-0315 and SM-102 ionizable lipids as analyzed by unpaired t-test. Error bars represent SD between duplicate samples, with significant changes represented by **** ($p<0.0001$) as assessed by an unpaired t-test.

3.2 Ring Micromixer Architectures and Variations

Although the demonstration of mixing and mRNA-LNP formation with serpentine channels was sufficient, it is well known that the implementation of "split and recombine" architectures, as in ring micromixers, which induce collisions in the flow path, enhance mixing profiles at low Reynold's numbers [10, 11]. Due to the popularization of ring micromixers, specifically the 4 ring micromixer, which split and recombine the flow to mix them, we began by implementing the use of a 4 ring micromixer. Prior to their operation, we screen the mixing efficiency using the sodium acetate buffer and absolute ethanol, to simulate the mRNA-LNP encapsulation process.

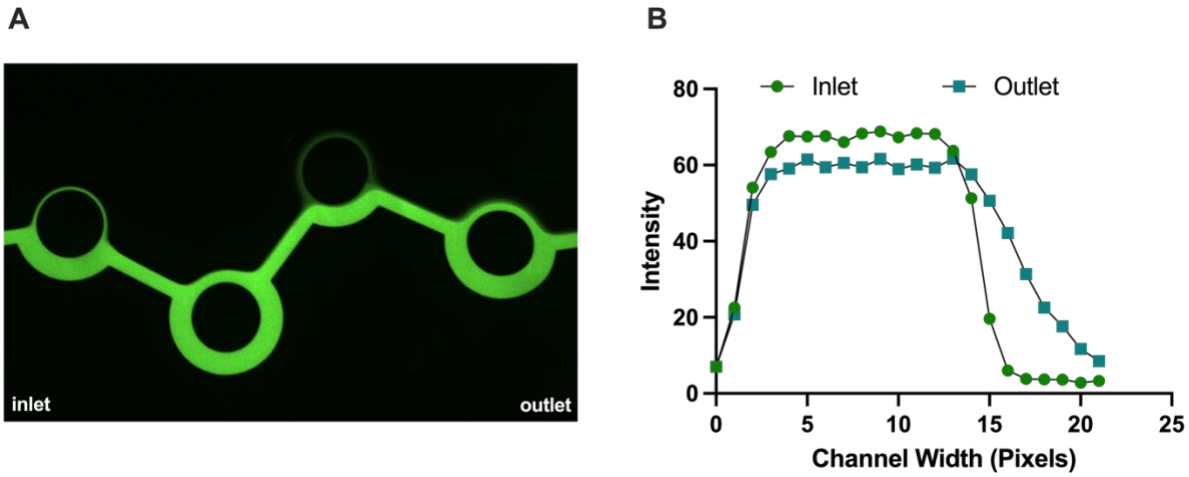

Figure 2.3. (A) Fluorescent imaging of the flow of acetate buffer and absolute ethanol streams at a total flow rate of 0.4mL/min (FRR=3) in a 4 ring micromixer. (B) Intensity profiles from the inlet and outlet of the microfluidic channels.

Microscopy images and intensity profiles revealed that although there is an increase in mixing from the inlet to the outlet (Figure 2.3), two distinct steams of fluid are observed side by side rather than a homogeneous mixture. Previous work has demonstrated that for 4 ring micromixers, a homogeneous flow profile was achieved at total flow rates above 4 mL/min [12]. These elevated flow rates, however, are not feasible for lab scale and developmental screening for LNP drug productions [13].

Based on these results, we evaluated the possible impacts of the addition of rings in order to extend mixing length and time and hypothesize that this may allow for increased homogeneity and potentially improve the encapsulation efficiency achieved. Here, we observe that despite the addition of rings, SM-102 maintains a consistent encapsulation efficiency of approximately 80% across 4 ring, 8 ring and 12 ring micromixers (Figure 2.4). However, in the case of ALC-0315, the addition of rings, specifically from 4 rings to 8 rings, increases the mixing efficiency and leads to greater EEs (Figure 2.4D). Again, we observed a lack of flow rate dependency on SM-102s ability to complex with the mRNA provided in the mixture in all variations of the ring micromixers that we evaluated.

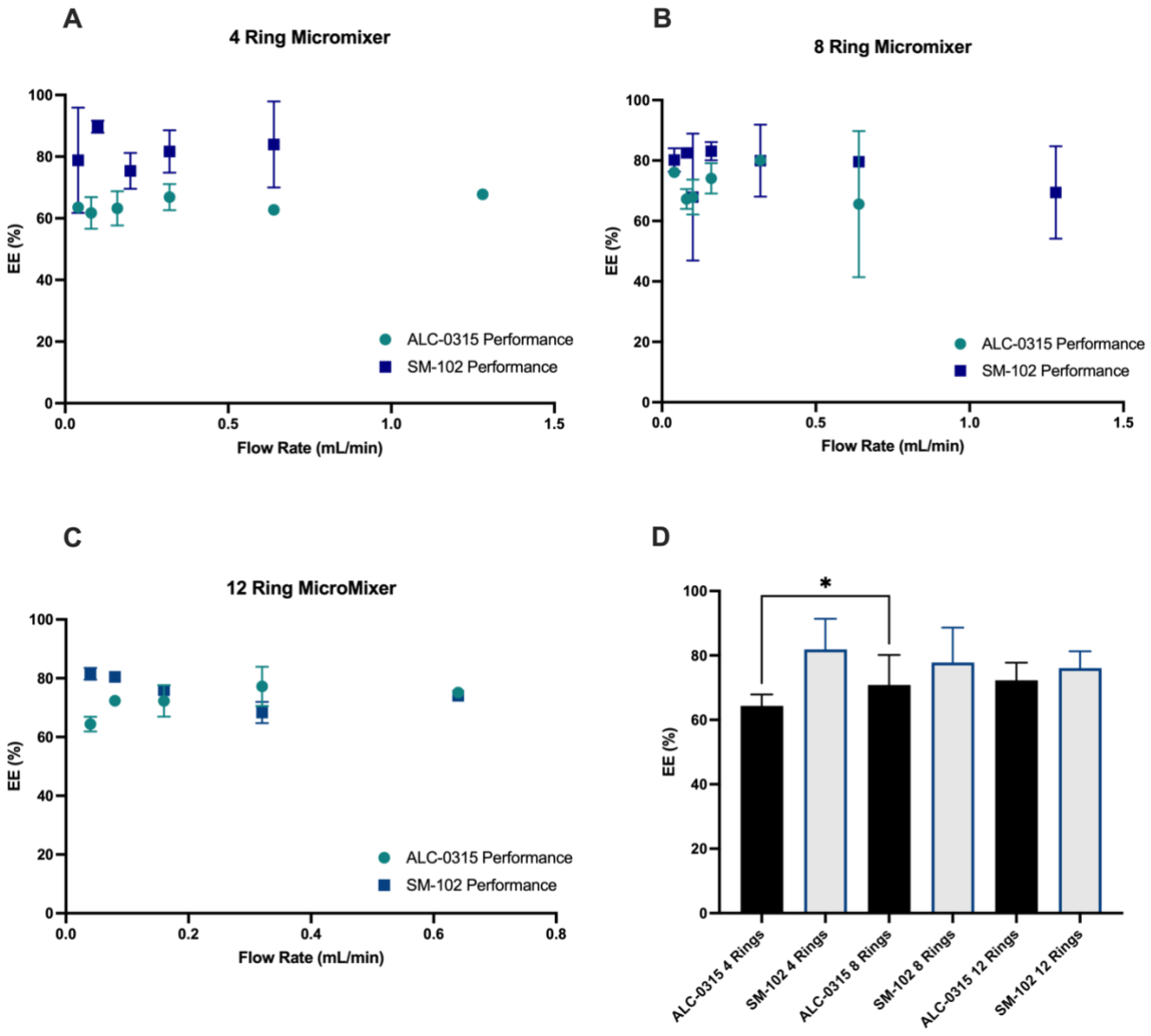

Figure 2.4. Encapsulation efficiency achieved as a function of flow rate in (A) 4 ring micromixer (B) 8 ring micromixers and (C) 12 ring micromixers (Error bars represent standard deviation between duplicate

samples). (D) Mean values of encapsulation efficiency across all flow rates classified by device and ionizable lipid with significant changes ($p < 0.05$) denoted with *.

As a response to the improvement observed in the encapsulation efficiency of ALC-0315 based LNPs after an increase to the 8 ring micromixer, we evaluated the impact of this modification on the size and PDI of the particles. Figure 2.5 demonstrates that in both cases, the acceptability criteria for both the size and PDI of the particles are met. Furthermore, we observe that in the case of the 8 ring micromixer, sizes of the particles are slightly larger at each flow rate, perhaps due to the additional time given to the lipids to accumulate and form the LNP in this case.

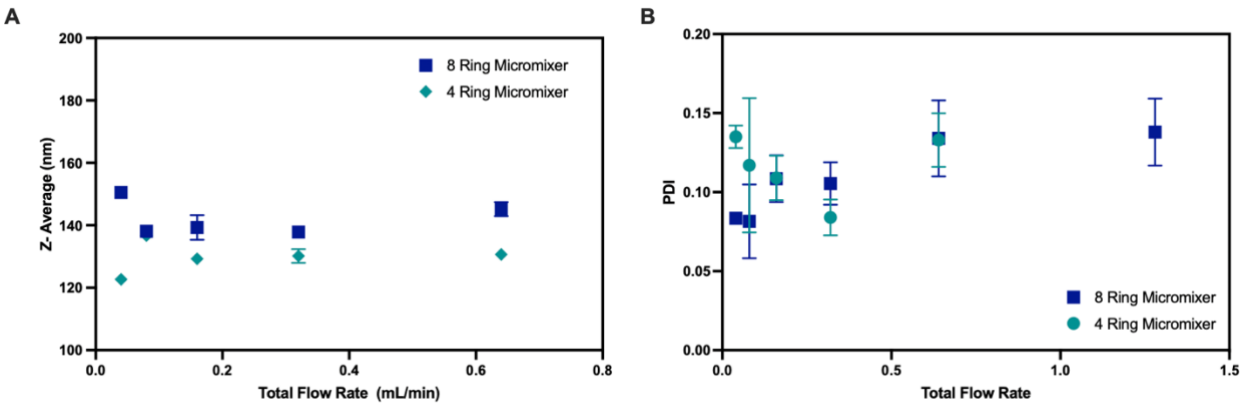

Figure 2.5. (A) Comparison of size measurements of ALC-0315 based mRNA-LNPs produced by 8 ring micromixers and 4 ring micromixers and (B) Comparison of their PDIs.

3.3 Obstruction Mixer Architectures and Variations

Having observed the success of both the 4-ring and 8-ring micromixer, we designed passive obstruction micromixers, which include rectangular obstacles in the flow path, creating a rectangular wave pattern, to induce flow patterns which will force both streams to collide and mix more efficiently. Previous numerical simulations have presented data which supports the use of these square wave based mixers over ring micromixers [14]. Two length variations of these devices were tested, one constituting 12 obstructions and another constituting 24 obstructions. The mixing indices of these devices have been shown to increase with the number of obstructions present [14], therefore both variations were studied to assess the impact on mRNA-LNP formulation.

We found that these devices were successful at encapsulating mRNA-LNPs and satisfied the acceptability criteria in the case of SM-102 (Figure 2.6A, 2.6B). However, in the case of ALC-0315, encapsulation efficiencies consistently remained below 80% (Figure 2.6A, 2.6B). A slight increase was observed in the mean encapsulation efficiency for ALC-0315 based particles across all conditions when the longer obstruction mixer was used (Figure 2.6C). Due to this improvement, and prior evidence of improved mixing indices at increased lengths, we further investigated the particles produced by the long obstruction mixer (24 obstructions). Our results demonstrate that at all conditions, the acceptability criteria for size and PDI are met, with the particles varying in size from around 167nm to 140nm based on the flow rate (Figure 2.6D).

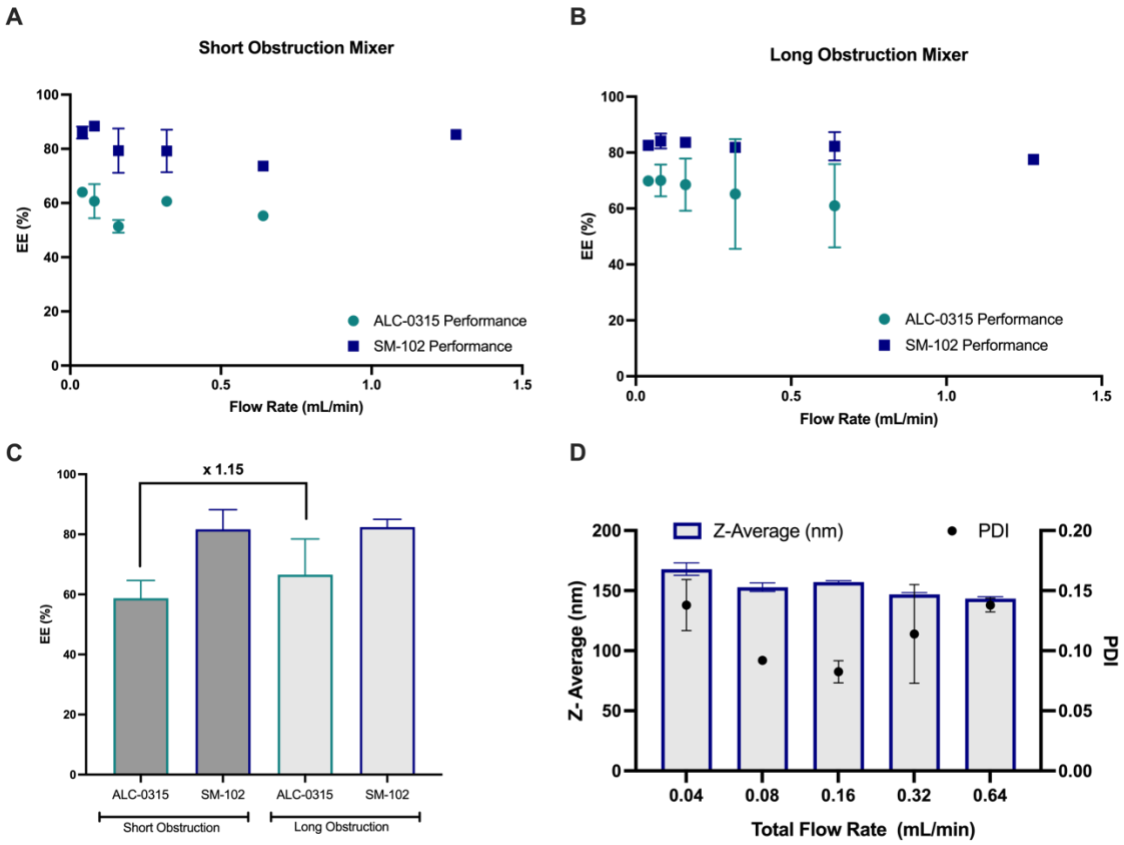

Figure 2.6. (A) Encapsulation Efficiency as a function of flow rate for short (12 obstructions) mixer and (B) long (24 obstructions) mixer, (C) Mean encapsulation efficiencies across all flow rates assessed classified by ionizable lipid and type of mixer used and (D) Sizes and PDI of ALC-0315 mRNA-LNPs produced using the long (24 obstructions) mixer.

3.4 In-House Microfluidics Present Feasible and Cost Effective Measure for mRNA-LNP production

Overall, we present simple and effective microfluidic mixing methodologies for mRNA-LNP formulation. A thorough data set has been compiled for the critical quality attributes (encapsulation efficiency, size and PDI) for both ring micromixers and obstruction mixers, two promising architectures for this application. Our data demonstrates that over a range of flow rates, these two microfluidic device designs fulfill the acceptability criteria of mRNA-LNP production for SM-102 based particles (Figure 2.7), without the need for expensive equipment or intensive technical training for their operation. Devices were manufactured via soft lithography (Figure 2.7A, 2.7D) and render efficiently loaded particles (Figure 2.7B, 2.7E) within a uniform size range reproducibly.

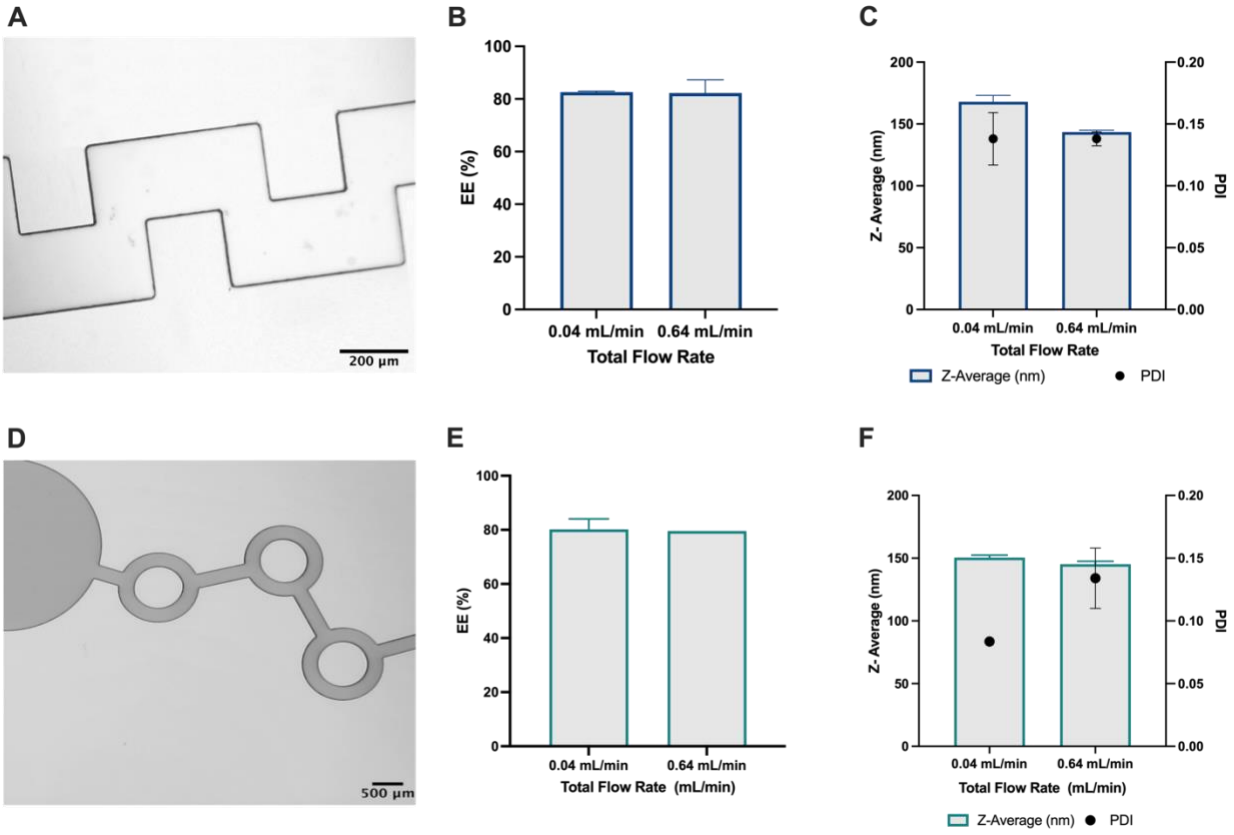

Figure 2.7. Summary of all critical quality attributes assessed demonstrating feasibility of the techniques. (A-C) illustrate the obstruction mixer and the quality attributes of the particles produced by it at a low (0.04 mL/min) and higher (0.64 mL/min) flow rate, (D-F) illustrate the ring micromixer and the quality attributes

of the particles produced by it at a low (0.04 mL/min) and higher (0.64 mL/min) flow rate. Error bars represent SD between duplicate samples.

4 Discussion

With an increase in interest surrounding mRNA-LNP therapeutics and vaccines, many academic laboratories, as well industrial institutions require scalable manufacturing technologies which are also amenable to the discovery phase. We developed simple, two-dimensional microfluidic designs, and conducted an analysis on their performance in terms of their encapsulation efficiency, size and polydispersity index, providing an economical alternative to the commercial automated benchtop microfluidic LNP assemblers.

We first assess the impact of the ionizable lipid on the results of encapsulation efficiency. Our results demonstrate that the ALC-0315 lipid, implemented in the Pfizer BioNTech COVID-19 mRNA vaccine, consistently produced LNPs with lower EE (%) than SM-102, implemented in the Moderna mRNA vaccine. These results are consistent with previous reports which have shown that SM-102 produces particles with higher EEs [15]. This demonstrates that although the manufacturing method impacts this quality attribute, the encapsulation efficiency is largely influenced by the ionizable lipid's ability to interact electrostatically with the negatively charged mRNA molecule, and thus these lipids should be optimized alongside their intended manufacturing method. Researchers have hypothesized that SM-102 allows for improved complexation with RNA due to its high pKa value of 6.75, meaning that more SM-102 molecules would be in the protonated state during the encapsulation [16]. Furthermore, we can also hypothesize that these differences can be attributed to the fact that ALC-0315 was produced for a manufacturing process which implemented the use of turbulent jet mixing [17]. Therefore, this lipid may only be more efficient in flow regimes with higher Reynold's numbers, to enable chaotic mixing. We observe an inverse effect with SM-102 based particles, whose encapsulation efficiency did not vary widely across the devices tested, and across the flow rates which were examined. This indicates that the electrostatic interaction of SM-102 with mRNA molecules is not necessarily reliant on the mixing efficiency.

Next, we examined the use of four ring micromixers, as well as extended versions of this design. This design has been implemented in the Precision Nanosystems NanoAssemblr Platform, which has been widely adopted by the field [18]. We find that the inclusion of four additional rings improves the performance of ALC-0315 in its complexation with mRNA. Due to the low flow rates adopted in the study, and the corresponding low Reynolds numbers, there is a limitation in the advection transport which allows for mixing in these micromixers. In these cases, increasing the length of the mixing channels aids to improve mixing efficiencies, despite the low flow rates [19]. Maintaining these low flow rates in the study was critical in order to ensure that the volume requirements for their operation remained low (<1 mL) to serve screening purposes. Future studies can explore the use of "unbalanced" rings, where the width of the rings is unequal on either side, in order to induce unbalanced collisions and enhance the mixing performance of the devices [10].

Lastly we introduce the obstruction mixer's ability to produce mRNA-LNPs of comparable quality to the ring micromixers. Similar devices have been implemented for mixing applications outside of the mRNA-LNP space [14], as well as by Kimura et al. for the production of LNPs [20]. They find that a minimum number of 10 obstructions is necessary for the production of controlled LNPs, whereas we propose the inclusion of an increased number of obstruction so as to increase the residence time and thus the mixing efficiency at low flow rates [20]. The simplicity of these devices can eventually allow for their integration into continuous manufacturing platforms, which would include the generation of mRNA within the microfluidic channels prior to encapsulation [21]. We can also consider other manufacturing methods of these devices to further extend the range of flow rates available to the users. Specifically, glass based microfluidics are expected to be able to withstand higher pressures due to their rigidity, improving the flexibility and performance of these devices [22].

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Preface to Chapter 3

Chapter 2 demonstrates our ability to effectively produce mRNA loaded lipid nanoparticles through cost-effective methods. The presented methodologies aim to improve accessibility of these technologies and provide data on suitable conditions for mRNA-LNP manufacturing which meet all proposed acceptability criteria.

Throughout the work involved in this chapter, we establish the necessary analytical techniques necessary to monitor the quality of the produced LNPs, including the Ribogreen assay as well as the use of the Dynamic Light Scatterer. These techniques are imperative for the work presented in the following chapter, which involves the close monitoring of LNPs over the course of time and other stresses. Furthermore, this chapter served to establish the preparation of mRNA-LNP materials within the Kamen lab, allowing us to pursue the work involved in the following chapter.

To further improve the accessibility and to facilitate the implementation of these products, both within our environment at McGill University as well as overall global distribution, the thermostability of mRNA-LNPs must be further improved. Chapter 3 delves into possible strategies and formulations to overcome this challenge. It explores the possible degradation methods of mRNA-LNPs and emphasizes the need for the balance required to maintain both the mRNA, as well as the LNP over long term storage periods.

Together, both Chapter 2 and 3 provide a working foundation to enable the manufacturing of these vaccine and gene therapy products in quantities which can not only sustain screening studies but also larger clinical studies and establish the appropriate assays to effectively do so. Furthermore, the efforts to improve the accessibility of these products contribute to the pandemic preparedness initiatives which were considered with the conception of this project.

Chapter 3: Across Time and Temperature: Evaluating the Stability of mRNA-LNPs through Freeze Thaw Stresses and Long Term Storage

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1 Introduction

The approval of mRNA drug products has been accompanied by the popularization of ionizable lipid nanoparticles for delivery. Due to the inherent instability of mRNA, its clinical applicability was limited without the use of a protective layer to prevent the RNA's enzymatic degradation and to facilitate its entry into the cytoplasm. LNPs expedited these products into the market by overcoming the extra-cellular and intra-cellular barriers associated with the RNA delivery. Thus, these delivery vehicles have been attributed a large portion of the success of the COVID-19 vaccines [1].

However, despite their success, LNPs have become associated with thermal instability and a reliance on ultra-cold storage conditions [2, 3]. Many degradation mechanisms for mRNA-LNPs have been described, primarily their physical degradation, including the aggregation or fusion of particles [4]. Furthermore, several chemical degradation pathways, such as hydrolysis and oxidation of both the mRNA and lipid species have been observed. These challenges impose hurdles in the acceleration of the development of non-urgent mRNA-LNP drug products. Though it is a growing field, there is a current lack in the number of mRNA-LNP temperature and stability data in publications and there remain many opportunities to improve the platform and to fill existing knowledge gaps. As of now, currently approved mRNA-LNP products have claimed limited stability at refrigerated and room temperatures (Table 3.1), hindering the distribution of these vaccines [2].

Vaccine Name	Ionizable Lipid	2-8°C Shelf Life	Room Temp Shelf Life	Concentration
BNT162b2	ALC-0315	Up to 5 days	Up to 2 hours	6-10 doses per vial
mRNA-1273	SM-102	30 days	Up to 12 hours	5-20 doses per vial

Table 3.1. Claimed stabilities of mRNA-LNP vaccines approved for use during the SARS-COV-2 pandemic. Table adapted from [2] and modified using [5, 6].

Furthermore, in pandemic situations, such as in the case of SARS-COV-2, mRNA vaccines were stored in multi-dose concentrations, which aided in maintaining the physical stability of the particles and were diluted prior to administration [7]. However, for therapeutic and non-urgent applications, there is a reduced feasibility of employing high LNP storage concentrations. Achieving stability at reduced LNP concentrations is imperative to enable the transition of mRNA-LNP products into the therapeutic field.

To this end, two main methods to enhance lipid nanoparticle stability have been explored. The first method is through iterative lipid design, by introducing changes in the lipid chemical structures [8]. However, these studies require extensive testing of lipid libraries, and their conclusions cannot be easily integrated into previously approved products or mRNA therapeutics currently in development. Otherwise, it is possible to optimize the formulation of the buffer and additional excipients to act as protective agents throughout the storage, and stresses involved in the delivery of mRNA vaccines and therapeutics.

Herein, we evaluate the physicochemical stability (size, polydispersity index, encapsulation efficiency) of mRNA-LNPs in the presence of several excipients, both throughout freeze-thaw stresses and multi-week storage at reduced LNP concentrations. The study aims to enhance the available data on LNP stability and improve our understanding of these particles' behaviors across different temperatures and time points. Furthermore, we aimed to identify excipients which aid in the preservation of mRNA-LNPs.

2 Material and Methods

2.1 mRNA Synthesis and Purification

A DNA construct encoding for eGFP (pGEM4Z-EGFP) was purchased from Addgene and a construct encoding for nLuciferase (pcDNA-LUC-CBR2opt-T7AG-C1) was gifted by the National Research Council of Canada (NRC). *In-vitro* transcription of the two DNA constructs was conducted using the MEGAscript T7 Transcription Kit (Invitrogen) following the manufacturer's protocol. The resulting mRNA was purified by phenol-chloroform purification and quantified

using the Quant-it Ribogreen RNA Assay Kit (Invitrogen). Final mRNA sequence length and purity was verified by formaldehyde gel electrophoresis in 1x MOPS buffer.

2.2 Lipid Nanoparticle Formulation

mRNA- LNPs were formulated by rapid pipette mixing of the ethanol and aqueous phases. In the ethanol phase, the lipids were resuspended in 100% ethanol at a molar ratio of 50: 10: 38.5: 1.5 (ALC-0315: 1,2-DSPC: Cholesterol: ALC-0159) to a total of 1 mg/mL. ALC-0315 and ALC-0159 were acquired from Cayman Chemical, while Cholesterol and 1,2-Distearoyl-sn-glycero-3-phosphocholine were acquired from Sigma Aldrich. For the aqueous phase, the mRNA was diluted in 50 mM sodium acetate (pH 5.0). The lipid to mRNA weight ratio was maintained at 10:1 for all experiments. After formulation, mRNA-LNPs were dialyzed using the Slide-A-Lyzer™ Dialysis Cassettes, 10K MWCO in 1000 volumes of either 1x PBS or 0.1M Tris buffer for at least 2 hours at 4 °C.

2.3 Freeze Thaw Studies

After dialysis, LNPs were diluted with appropriate excipients. The trehalose was obtained from Fisher Bioreagents, the mannitol was obtained from BioBasic, the Kolliphor P188 was obtained from Sigma Life Science, the Pluronic F127 was obtained from Sigma. Samples were frozen in 1.5 mL plastic Eppendorf tubes at -20°C for at least 24 hours between each thaw. Samples were thawed at room temperature for 30 mins prior to characterization.

2.4 Long Term Storage

After dialysis, nLuciferase encoding LNPs were characterized then diluted with the appropriate excipients The trehalose was obtained from Fisher bioreagents, the mannitol was obtained from BioBasic, the Kolliphor P188 was obtained from Sigma Life Science, the Pluronic F127 was obtained from Sigma. 100 µL aliquots were frozen in 1.5 mL plastic Eppendorf tubes and were only thawed once at the time of analysis. Samples were thawed at room temperature for 30 mins prior to characterization.

2.5 mRNA-LNP Characterization

RNA encapsulation efficiency (EE%) of the LNPs was determined by a modified Quant-it Ribogreen RNA Assay Kit (Invitrogen) protocol. Duplicate LNP samples were diluted 1:50 in either 1X TE buffer, to determine the unencapsulated RNA concentration, or in 2% Triton X-100, to determine the total RNA concentration, in a 96 well plate. A standard curve was prepared using the Ribosomal RNA standard provided in the kit. The plate was incubated for 10 min at 37°C to disrupt LNPs prior to the addition of Ribogreen Reagent into each well and the fluorescence intensity values were measured by the Agilent BioTek Synergy HTX MultiMode Microplate Reader. EE% was calculated as the difference between the total RNA concentration and the unencapsulated RNA concentration divided by the total RNA concentration. Size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using the ZetaSizer Nano S90 (Malvern Panalytical). LNPs were brought to room temperature and diluted 1:15 in PBS in a 50 µL disposable cuvette (SARTEDT). The material refractive index (RI) was set to 1.37, the and the temperature was maintained at 25°C. Each sample was subjected to 2 runs of at least 10 measurements each. Percent changes of the quality attributes assessed were calculated as described in equation 1.

$$Percent\ Change\ (\%) = \frac{(Final\ Attribut\ Value - Initial\ Attribute\ Value)}{Initial\ Attribute\ Value} \times 100 \qquad \text{Eqn. 1}$$

2.6 Verifying mRNA integrity

mRNA-LNP samples were treated with 0.1% Triton and incubated at 37°C for 10 mins to break open particles at the 2 month storage mark. Samples were diluted 10-fold and heated at 70 °C for 2 minutes prior to performing electrophoresis with the 2100 Agilent Bio system using mRNA pico chip.

3 Results

3.1 Freeze Thaw Screening

In order to evaluate mRNA-LNP stability, ALC-0315 was chosen as the ionizable lipid to begin due to its clinical relevance, having been included in an FDA approved vaccine, and due to its known lack of thermostability. Additionally, formulations in Tris buffers were evaluated as there has been evidence that this buffer is superior to PBS in the protection of mRNA-LNPs [9]. Table 3.2 summarizes the Tris formulations which were investigated. It is known that in the absence of excipients and cryoprotectants, mRNA-LNP formulations are unstable and prone to physical degradation. As such, it was expected that the inclusion of a surfactant and sugar would reduce aggregation and degradation through freeze thaw stresses as compared to an unformulated control.

Buffer	Surfactant	Sugar	Formulation
0.1M Tris (pH 8)	0.5% P188	8% Sucrose	Tris F1
		8% Trehalose	Tris F2
		8% Mannitol	Tris F3
	0.5% F127	8% Sucrose	Tris F4
		8% Trehalose	Tris F5
		8% Mannitol	Tris F6
	None	None	Tris Control

Table 3.2. Tris containing formulations evaluated in the initial freeze thaw screening. All formulations contained a surfactant and sugar as excipients except for the control.

Figure 3.1 illustrates the results of the change in particle quality attributes prior to and after thawing them 5 times. It is evident that when no excipients are present, the polydispersity index increases drastically, exceeding an 80% increase in this condition. Moreover, in all of the examined cases, an increase in the size was observed. This increase in size can indicate the swelling or aggregation of the particles, which is a known consequence to temperature cycling [10].

Based on a global evaluation of the change in EE, change in Z-average, and change in PDI, the Tris based formulations which demonstrated the least amount of change after 5 freeze thaws were Tris F1 (0.5% P188, 8% Sucrose) and Tris F6 (0.5% F127, 8% Mannitol). Both of the

aforementioned formulations significantly reduced changes observed in polydispersity index observed over time. Furthermore, as compared to the control, they did not significantly vary in terms of changes in the encapsulation efficiency and size.

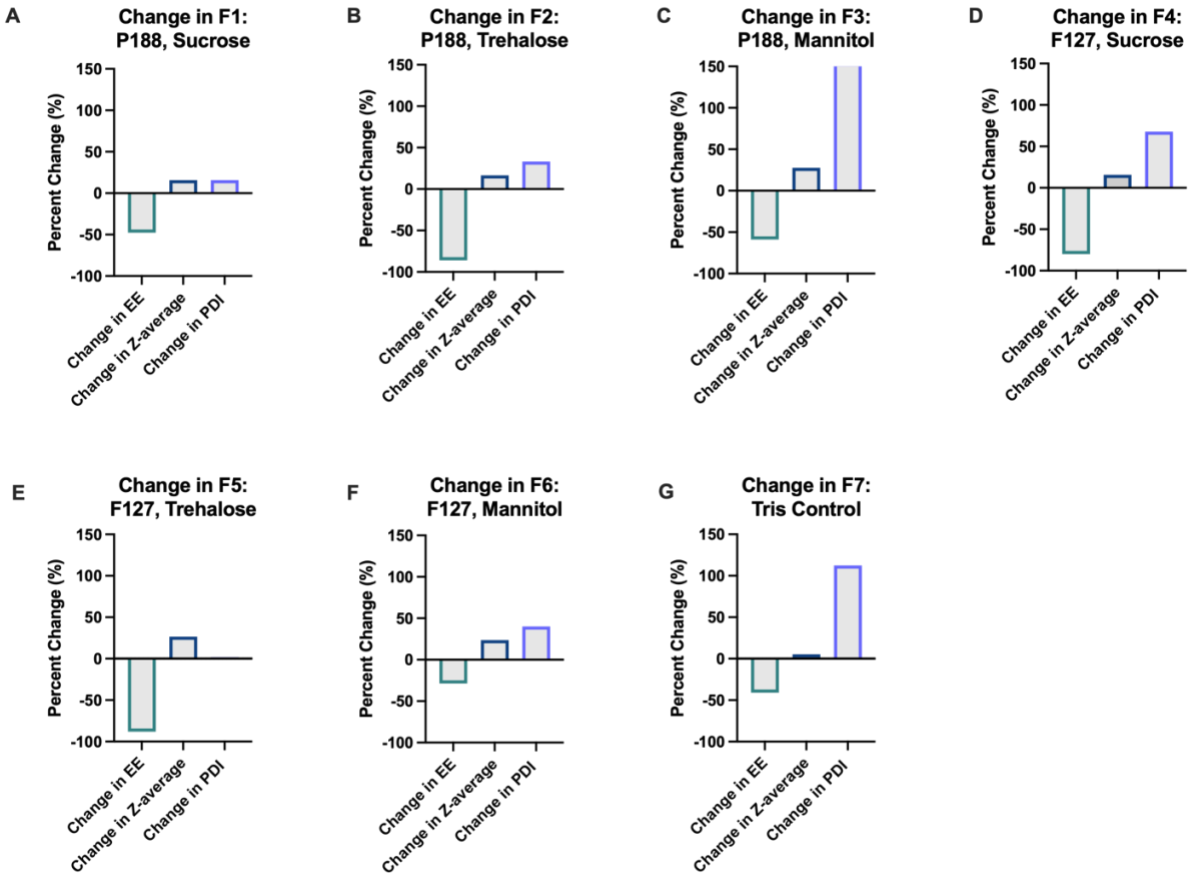

Figure 3.1. Change in the encapsulation efficiency (EE), size and polydispersity index (PDI) of nLuciferase encoding mRNA-LNPs in Tris containing formulations after 5 freeze thaw cycles at -20°C. Changes are represented as a percent change as compared to their initial values. Value for PDI change in (C) exceeds axis but is maintained in this format to facilitate comparison between formulations. Thaw by Thaw data shown in Supplementary Figure S1.

To demonstrate the applicability of the identified formulations, we repeated the experiments with mRNA LNPs encoding eGFP rather than nLuc and observed consistent results, indicating that after 5 freeze-thaw cycles, less change was observed in the particles' as compared to the Tris only control (Figure 3.2). Specifically, it was once again noted that the excipients were capable of reducing the elevated polydispersity index that resulted from the repeated freeze thaw cycles in

the Tris only formulation. The PDI index remained below 0.2 for both formulated groups, indicating uniformity among these samples.

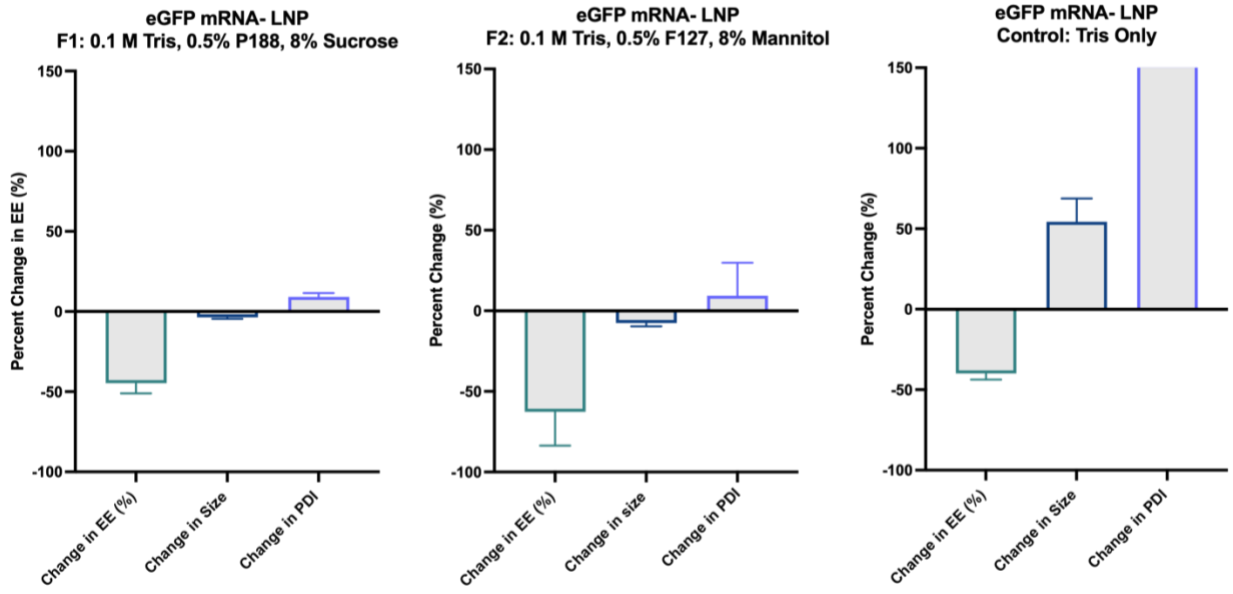

Figure 3.2. Change in the encapsulation efficiency (EE), size and polydispersity index (PDI) of eGFP encoding mRNA-LNPs after 5 freeze thaws at -20°C. Changes are represented as a percent change as compared to their initial values. Error bars represent the standard deviation between duplicate samples. The axis of (C) is cut off for ease of comparison.

3.2 Eight Week Storage Evaluation

To further explore the formulations screened through freeze thaw studies, we evaluated the stability of nLuciferase mRNA-LNPs under aqueous (Room Temperature and 4°C) and frozen conditions (-20°C and -80°C) over the course of two months. Having demonstrated that they are capable of withstanding temperature cycling and thermal stresses, the aim throughout the following section was to identify if they could withstand long term stresses. Samples were assessed at the 1 week, 4 week and 8 week marks. Both mRNA-LNPs formulated with SM-102 and ALC-0315 were observed throughout this time in order to expand the study. For this long term study, the two previously identified formulations are examined as well as tris only control (Table 3.3). Additionally, for this portion of the study, a supplemental assay was conducted to assess mRNA integrity since the physicochemical stability of mRNA-LNPs does not necessarily correlate with their biological activity [11].

Formulation Content	Formulation Name
0.1M Tris, 0.5% P188, 8% Sucrose	F1
0.1M Tris, 0.5% F127, 8% Mannitol	F2
0.1M Tris	Control

Table 3.3. Tris containing formulations evaluated during 8 week storage. All formulations contained a surfactant and sugar as excipients except for the control.

For all formulations, some losses in encapsulation efficiency or degradation of mRNA may be attributed to the lack of capping on the RNA which was produced. Furthermore, a singular size measurement was taken prior to addition of excipients and storage, as an initial time point for reference of size growth over time. Therefore, some size increases in the samples with excipients may be attributed to the addition of these excipients leading to a slight increase in their determined size (Supplementary Figure S2).

3.2.1 Long Term Room Temperature Storage

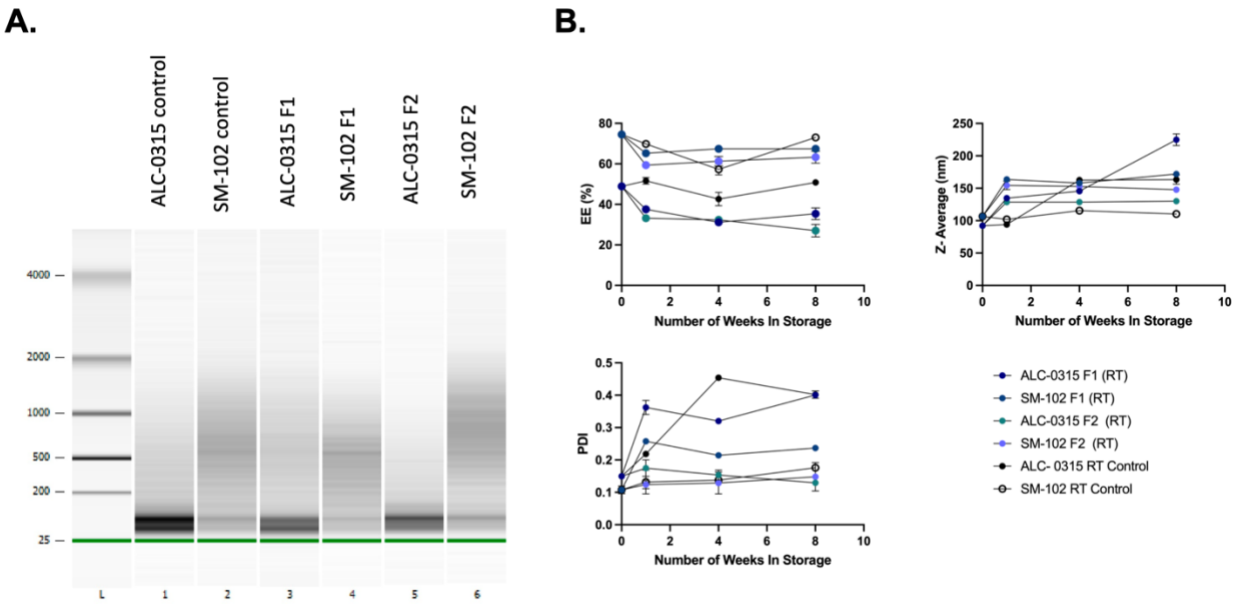

Figure 3.3. Stability Evaluation of mRNA-LNP samples stored at room temperature over the course of 2 months, with (A) demonstrating the mRNA integrity of the mRNA after 8 weeks and (B) demonstrating the evolution of the encapsulation efficiency (EE), the z-average and the PDI over the course of the storage duration. Error bars represent the standard deviation between measurements.

The stability of groups samples stored at room temperature was greatly impacted at room temperature. All samples stored at room temperature experienced near complete mRNA degradation (Figure 3.3A), as evaluated by the Bioanalyzer. Although the encapsulation efficiency did not decline significantly in all formulations tested (Figure 3.3B), the encapsulated mRNA within the particle appears to have degraded within 2 months of their storage and is thus non-functional.

Furthermore, we note that F2 (0.5% F127, and 8% Mannitol) was effective at limiting heterogeneity among the LNPs for both the samples formulated with ALC-0315 and SM-102 (Figure 3.3B). On the other hand, the sucrose and P188 containing samples exceeded a PDI index of 0.2 over the course of their storage at room temperature, demonstrating their inability to maintain the physicochemical qualities of the LNP in this storage condition.

Interestingly, the addition of excipients to the mRNA-loaded particles did not improve their stability at room temperature. Particles formulated with SM-102 ionizable lipid, in plain Tris buffer, experienced less changes in their quality attributes than their formulated counterparts (Figure 3.4).

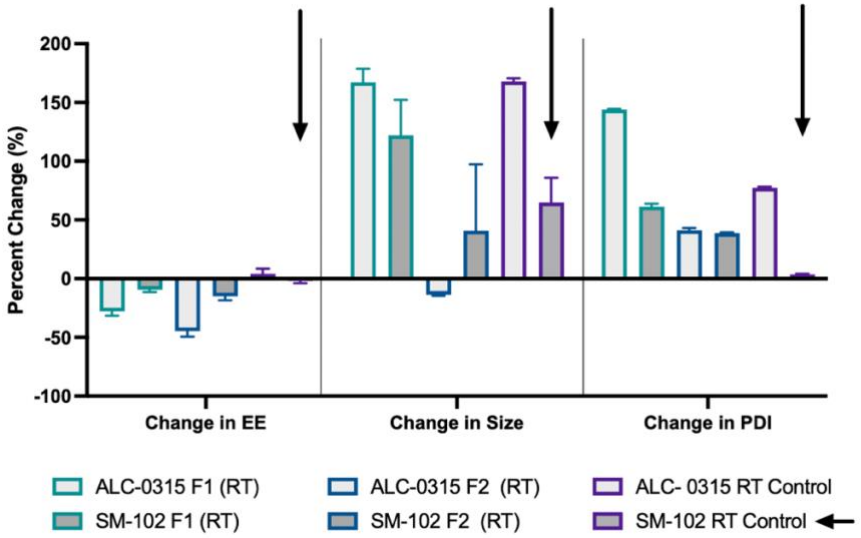

Figure 3.4. Overall changes experienced by nLuc mRNA-LNP particles after 8 weeks of storage at room temperature.

Additionally, the particles formulated with ALC-0315 were less consistently less stable, in terms of changes in their quality attributes, than those formulated with SM-102 at room temperature, no matter their buffer and excipient composition. In fact, observation of mRNA degradation between mRNA-loaded ALC-0315 and SM-102 particles reveals that the degradation is more severe in ALC-0315 particles at room temperature (Figure 3.3A). This emphasizes the role of ionizable lipids in maintaining the stability of mRNA-LNP vaccines and therapeutics, specifically as the field focuses its efforts on non-frozen storage conditions.

Overall, these results demonstrate that the challenge of storing mRNA-LNPs at elevated non-frozen temperatures primarily surround the instability of mRNA in this condition, rather than the instability of the physical lipid nanoparticle. This is made evident in cases where the particle's quality attributes are maintained over the course of two months of storage but the resulting mRNA from within the particle is no longer in its integral form. This emphasizes the balance that must be achieved to increase the shelf life of mRNA-LNPs since both components of the drug product require different conditions for their long term stability.

3.2.2 Long Term 4°C Storage

Following 2 months at 4°C, F2 (0.5% F127, 8% Mannitol) was identified as successful in maintaining mRNA integrity in SM-102 mRNA-LNPs as compared to the control particles (Figure 3.5A). This indicates that the additional excipients in this case aid in the preservation of the mRNA molecule. Additionally, the F1 buffer particles' physical qualities (size and PDI) were maintained similarly to the control throughout this 2 month storage period (Figure 3.5B, 3.5C, 3.5D). A size and PDI increase are observed in the F2 buffer within the first week of storage but remained stable in the weeks following.

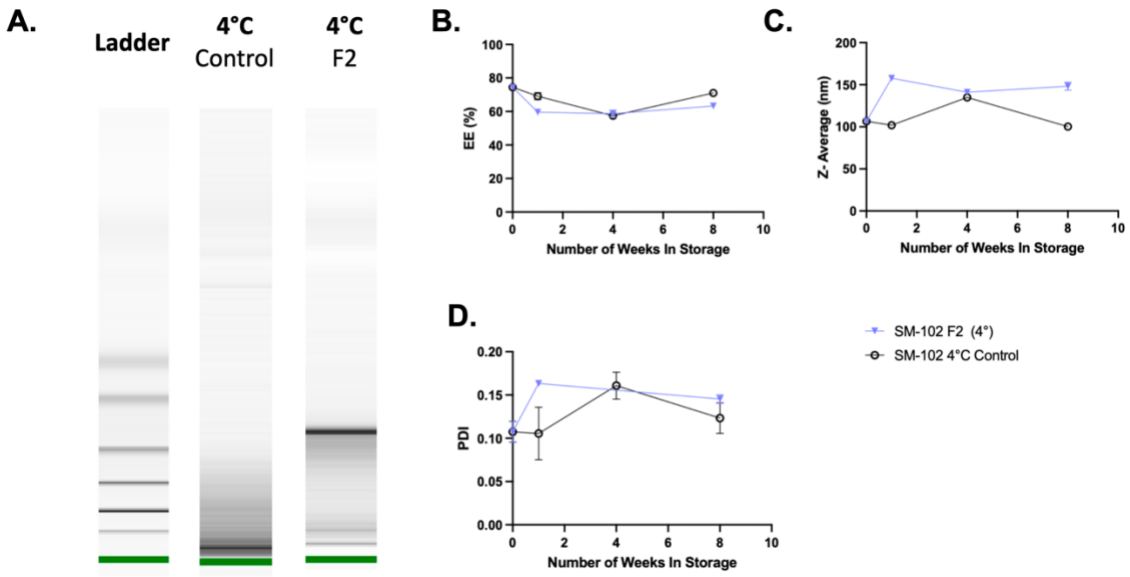

Figure 3.5. Stability Evaluation of SM-102 mRNA-LNP samples stored at 4°C over the course of 2 months, with (A) demonstrating the mRNA integrity of the mRNA after 8 weeks and (B) demonstrating the change of the encapsulation efficiency (EE), (C) the z-average and the (D) PDI over the course of the storage duration. Error bars represent the standard deviation between measurements. Controls represent samples in Tris buffer without additional excipients.

3.2.3 Long Term -20°C Storage

At -20°C, F1 (0.5% P188, 8% Sucrose) was deemed capable of maintaining the stability of ALC-0315 mRNA-LNPs as compared to the control particles after the 8 week duration of storage. A clear difference is observed in the mRNA integrity of the samples (Figure 3.6A). While no clear nLuciferase mRNA bands appear in the control mRNA-LNPs, a strong band remains in the sample which was preserved with sucrose and P188. Furthermore, it appears as though the addition of these excipients reduces the change in the polydispersity index of the particles over the course of 2 months of storage. Despite their growth in size, ALC-0315 LNPs stored in F1 for 2 months did not exceed the acceptable size criteria for mRNA-LNPs and were observed to grow to around 125nm in size.

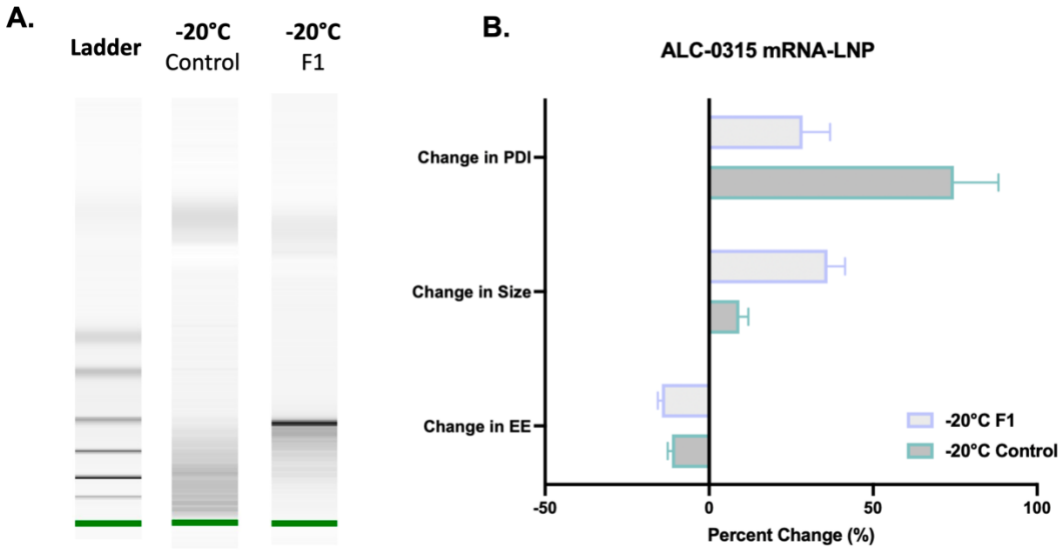

Figure 3.6. Stability Evaluation of ALC-0315 mRNA-LNP samples stored at -20°C over the course of 2 months, with (A) demonstrating the mRNA integrity of the mRNA after 8 weeks and (B) demonstrating the percent change of the encapsulation efficiency (EE), the size (z-average) and the PDI over the course of the storage duration. Error bars represent the standard deviation between measurements

3.2.4 Long Term -80°C Storage

Although storage at -80°C has been deemed a challenge for vaccine distribution, the formulations were studied at this temperature over the course of the two months in order to gain insight on the behaviour of mRNA-LNP particles in this condition. All SM-102 mRNA-LNPs stored during our study demonstrated a large decline in the encapsulation efficiency after one week of storage (Figure 3.7A). For both ionizable lipids, in storage conditions where a surfactant and sugar are not included, a dramatic increase in the size is observed within one week of storage at -80°C (Figure 3.7B). These unformulated samples also incurred the largest change in their PDI throughout their storage duration (Figure 3.7C). These results demonstrate the instability of the lipid nanoparticle shell without excipients at this temperature, and the need for the inclusion of excipients at -80°C to maintain the particles physical attributes and reduce aggregation. Despite a general growth of the particle size across conditions, both F1(0.5% P188, 8% Sucrose) and F2 (0.5% F127, 8% Mannitol) reduced the changes in size and PDI throughout storage, with F1 outperforming F2 (Figure 3.7B, 3.7C).

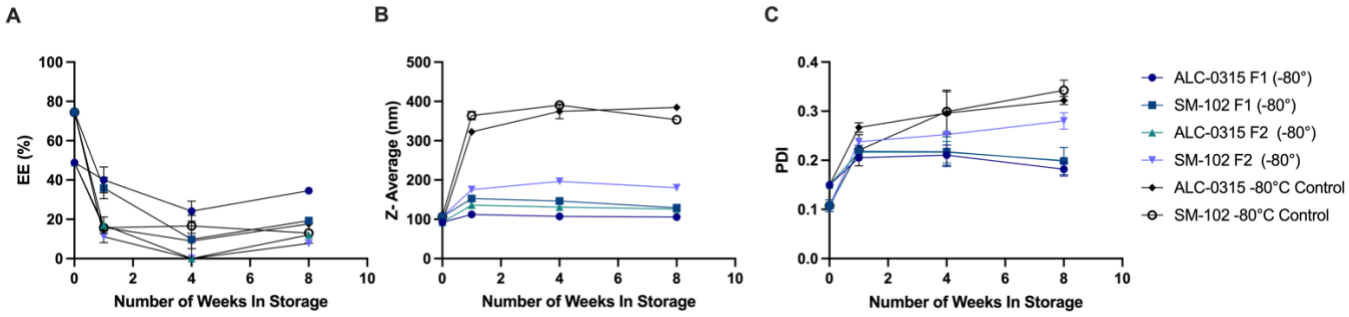

Figure 3.7. Change in the (A) encapsulation efficiency (EE), (B) size and (C) polydispersity index (PDI) of nLuciferase encoding mRNA-LNPs over the course of two months at -80°C. Error bars represent the standard deviation between duplicate measurements.

Despite the majority of particles experiencing a near complete degradation and decline in their encapsulation efficiency, one formulation demonstrated its ability to maintain the stability of the particle as well as retain mRNA in its integral form throughout the course of the 8 weeks during which they were studied (Figure 3.8). The addition of 8% sucrose and 0.5% P188 to mRNA-LNPs formulated with ALC-0315 minimized changes in their size and the polydispersity index as compared to the Tris only control. The encapsulation efficiency of these LNPs, stored with sucrose and P188, declined by approximately 29% over the course of 2 months at -80°C whereas those which were stored without a sugar or surfactant experienced a decline of approximately of 63.5% in their encapsulation efficiency. Furthermore, in addition to the preservation of the physical attributes assayed throughout their storage, a strong band of mRNA in its integral form is observed (Figure 3.8A).

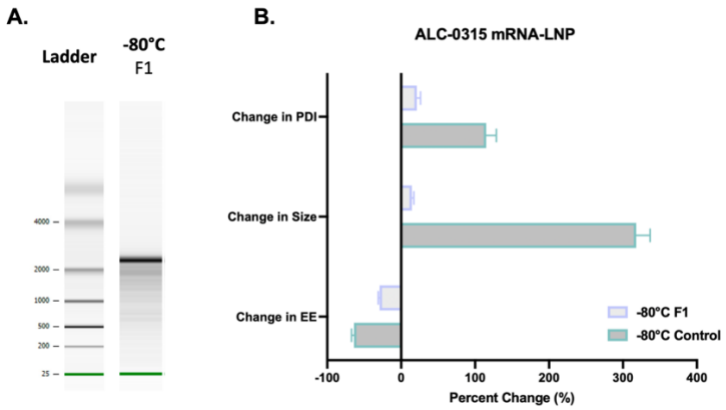

Figure 3.8. Stability Evaluation of ALC-0315 mRNA-LNP samples stored at -80°C over the course of two months, with (A) demonstrating the mRNA integrity of the mRNA after 8 weeks and (B) demonstrating

the percent change of the encapsulation efficiency (EE), the z-average and the PDI over the course of the storage duration. Error bars represent the standard deviation between measurements.

4 Discussion

mRNA-LNP vaccines and therapeutics have posed a large hurdle in their implementation due to their temperature sensitivity and general instability [12]. A primary limitation of the mRNA vaccines and therapeutics in the post pandemic market is this thermal instability. Specifically, as therapeutics and non-urgent vaccines roll out, dosages may now require longer term storage and may not need to be stored at high density multi dose concentrations. In this study, we screened six formulations based on their ability to minimize changes to the physicochemical characteristics of the ALC-0315 mRNA-LNPs after 5 rounds of freeze thaw at -20°C as compared to the particles which were unformulated (Tris buffer only). We found that a sucrose and P188 containing Tris buffer was effective at mitigating the losses incurred during freeze-thaw stresses. However, when P188 was replaced by F127, another surfactant, the mitigation effect was less prominent. This finding is consistent with findings that have indicated that P188, above a concentration of 0.0005% w/v, is an appropriate excipient to protect enveloped viral formulations throughout freeze-thaws [13]. Due to the limited data on thermostability of mRNA-LNPs, we can draw the comparison between mRNA-LNPs and enveloped viruses, since they are both comprised of a lipid-containing envelope which encapsulates genetic material. Additionally, we identified that the addition of Mannitol as a cryoprotectant along with surfactant F127 was effective in protecting the mRNA-LNPs throughout these thermal cycling stresses as compared to the control. This is in line with previous studies that have indicated that mannitol was a suitable additive to prevent aggregation during freezing [14]. Although some sugars and sugar alcohols have been evaluated as excipients for mRNA-LNPs, the combination of these excipients with surfactants have not been explored in publications to our knowledge. With the uprising of concern against other respiratory diseases, and an interest in intranasal delivery, the introduction of surfactants into vaccine and drug formulations is typical for nebulization [15].

Under a stress condition of room temperature storage, all formulations failed to maintain mRNA integrity. This result is unsurprising as it has been well documented that mRNA itself is unstable in aqueous conditions due to hydrolytic cleavage of the phosphodiester backbone of the RNA [16].

Previous research has indicated that within the LNP core, approximately 20% of the volume is accounted for by water, implying that the mRNA is exposed to this hydrolysis even while encapsulated [4]. Furthermore, at elevated temperatures, the relative humidity of the air has been shown to degrade RNA, and thus this humidity would need to be more strictly controlled for room temperature storage of these molecules [16]. Furthermore, ribonucleases are known to be increasingly active at elevated temperatures [16]. However, despite mRNA degradation, the physicochemical characteristics of the LNPs were maintained in some of the cases tested, even when stored without excipients. This implies that the instability of mRNA remains the challenge to be surpassed to extend mRNA-LNPs room temperature shelf-life. This is further supported by the specified shelf-life of Onpattro, an siRNA-LNP drug, which can be stored at room temperature for 14 days or be refrigerated for up to 3 years [17, 18]. It has been noted that the LNP composition of Onpattro is similar to that of the mRNA vaccines, indicating that the limiting factor for storage at ambient temperature is limited by the inherent instability of the mRNA. In contrast to mRNA-LNPs, this siRNA-LNP, should not be allowed to freeze throughout its storage duration, most likely to maintain the physical properties of the LNP [19]. Our results emphasized these conflicting needs and stabilities of the lipid nanoparticle component from the mRNA component.

In an effort to achieve this aforementioned balance, after 8 weeks of storage at 4°C, -20°C, and -80°C, we identified an improved storage condition for each of these temperatures as compared to the controls. At 4°C, the inclusion of mannitol and F127 to SM-102 based lipid nanoparticles demonstrated improved mRNA integrity retention as well as comparable size and PDI stability to the Tris only control. This represents a progression towards the extension of the 4°C shelf life of SM-102 based LNPs, which was listed as 30 days [2]. At -20°C and -80°C, our results demonstrated that ALC-0315 based LNPs were increasingly stable with the addition of sucrose and P188, both in terms of LNP physicochemical characteristics as well as in terms of mRNA integrity. The ability to store ALC-0315 LNPs at -20°C for 2 months represents an improvement upon the ultra-cold storage requirements (-80°C to -60°C) of the BNT162b2 vaccine, which posed many challenges in their shipping and distribution due to this requirement [12]. Furthermore, the results of our freeze thaw study demonstrate that these excipients were also effective in maintaining the physicochemical characteristics of the LNP throughout several freeze-thaw cycles from -20°C to room temperature, which would ease restrictions on their shipping conditions.

In spite of having identified an improved excipient formulation at -80°C, this condition proved to be a harsh condition for the maintenance of LNPs. In the inverse case of room temperature, mRNA stored at -80°C maintained its integrity in most of the samples which were tested. However, we observed a decrease in the encapsulation efficiency of most particles, indicating leakage from or degradation of the particles. Additionally, we observed size increases and drastic increases in PDI in cases without the inclusion of a cryopreservative, indicating aggregation of particles. This is consistent with the results of Kamiya et al. who observed that in mRNA-LNP samples stored at -80°C, the encapsulation efficiency decreased 41.74% and the size increased 245.2% as compared to those stored at 4°C [20].

In conclusion, we provide data on the freeze-thaw dynamics and long term storage of mRNA-LNPs, in an attempt to answer remaining questions on their stability with the surge in public interest on mRNA-LNP vaccines and therapeutics. We identify potential formulations which balance stability of the mRNA with the stability of the LNPs, at 4°C, -20°C, and -80°C. These formulations include surfactants to facilitate eventual nebulization of the particles. We aim to increase the accessibility of these drug products through these findings. Future studies may assess lyophilized formulations for room temperature storage due to the difficulties incurred when storing mRNA-LNPs at room temperature in aqueous conditions.

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Supplementary Materials

Figure S1. Progression of nLuciferase encoding mRNA-LNPs quality attributes over the course of 5 freeze-thaw cycles at -20°C. Points represent the mean of duplicate sample measurements. Formulations F1-F7 are as specified in Table 2.

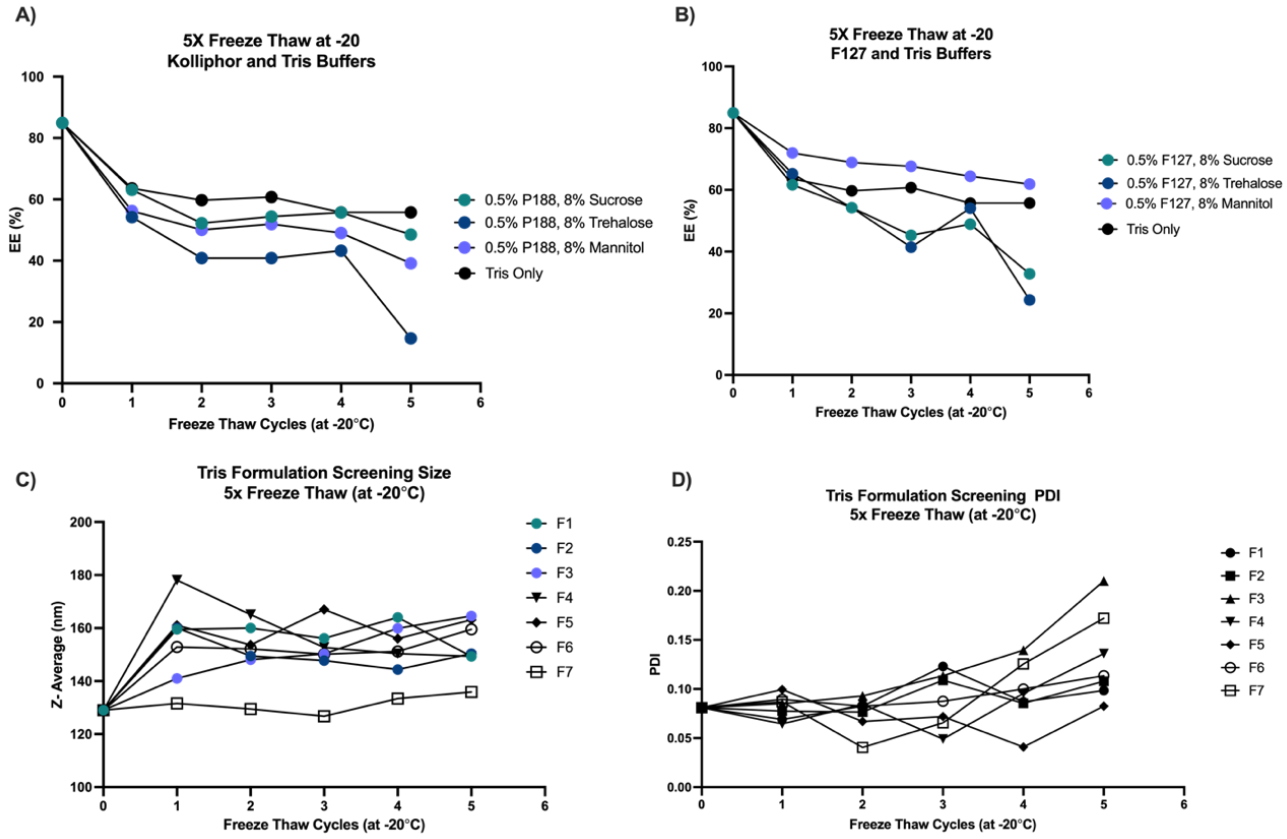
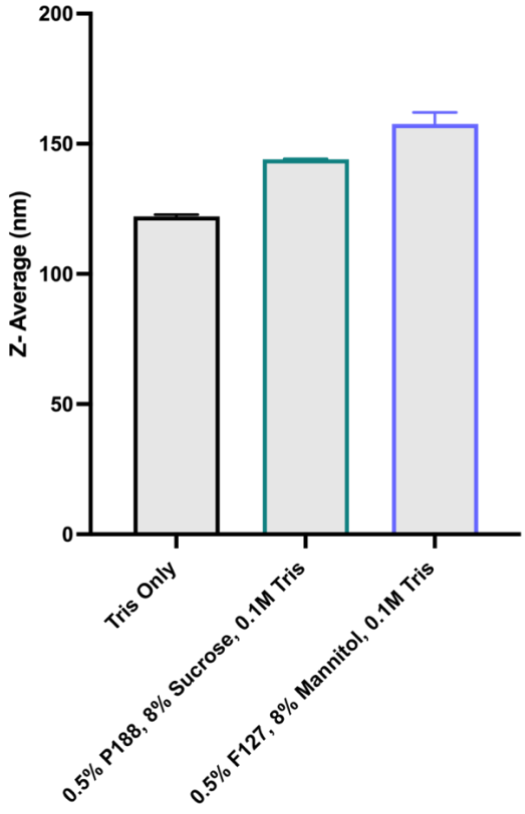


Figure S2. Demonstration of the change in DLS size measurement as a result of the addition of excipients. Samples shown represent eGFP encoding mRNA-LNPs produced in the same batch and dialyzed against 0.1M Tris together.



General Discussion and Conclusions

As a result of the COVID-19 pandemic, mRNA-LNP technology has gained notoriety and has shown immense therapeutic and immunotherapeutic potential in the past several years [125]. As such, efforts to implement this technology, both globally and locally, within McGill University, have seen a drastic increase. The present work contributes to establishing a foundation for the production and storage of mRNA-LNPs to enable this technology. This project aimed to develop and provide a cost effective method to manufacture mRNA-LNPs which adhere to quality standards and to improve the storage and accessibility of these products. This work provides a process engineering perspective to address challenges present within this biomanufacturing field and contributes towards ongoing pandemic preparedness efforts by enhancing the data available concerning mRNA-LNP technology.

Messenger RNA can be supplied to patients to induce an antigenic response in the context of vaccination and immunotherapy, to provide patients with missing or defective proteins, to delivery gene editing machinery to correct critical mutations or to deliver chimeric antigen receptors to cells for CAR-T cell therapies. These products, however, experienced a delayed entry into the clinical space as compared to viral vector and protein subunit vaccines and therapeutics due mostly to the inherent instability of mRNA. However, a number of milestones in their development, including modified nucleotides and the development of ionizable lipid nanoparticles have addressed the limiting weaknesses of the technology [126]. Importantly, a major advantage of mRNA-LNPs is that the development of a single effective manufacturing process can be applied to many drug products, with the exception of the change in the coding region of the mRNA, rendering it a platform technology [4]. These types of platform processes, once approved, facilitate the regulatory approval process, and are especially useful in the face of possible emergent pandemics [4].

Chapter 1 of the present work reviews the inception of mRNA therapeutics and vaccines, as well as established the importance of lipid nanoparticles, specifically their role in the clinical translation of mRNA biologics. Furthermore, this chapter highlights the challenges associated with mRNA-LNP manufacturing. It is made evident that the technology is difficult to distribute and remains

inaccessible in some low and middle income countries (LMIC) where ultra-low freezing temperatures are not sustainable and electricity outages may induce freeze-thaw stresses in the mRNA-LNP products [127]. Furthermore, this chapter uses details from ongoing clinical trials to demonstrate the variable dosage needs of mRNA-LNPs. This underlines that there exists a lack of accessible methodologies for the reproducible manufacturing of mRNA-LNPs, with flexible operational abilities, capable of serving lab scale productions as well as larger scale productions.

Chapter 2 aimed not only to provide the Kamen Lab with an accessible and cost effective methodology for mRNA-LNP production, but also demonstrate the feasibility of these methodologies to produce adequate mRNA-LNPs. Here, we demonstrated that simple two-dimensional microfluidics were capable of forming SM-102 based LNPs that fit within all specifications, consistently outperforming ALC-0315 based LNPs in terms of their encapsulation efficiency. We also demonstrate that despite the success and widespread use of 4 ring micromixers, we can enhance the performance of ALC-0315 in its complexation with mRNA by introducing an additional 4 rings to extend the mixing length. Furthermore, we propose an additional passive mixing technique, using an obstruction mixer. All mixers examined within the work performed well, producing particles with ~80% encapsulation efficiency, approximately 150nm in size and a PDI which did not exceed 0.2. This work provides a basis for mRNA- LNP productions, which can be further iterated to improve or modify the critical quality attributes achieved if needed. Furthermore, this work presents advantages in terms of the cost as well as the minimum volume requirements for mRNA-LNP production, since tube lengths and syringe volumes can be customized, allowing for total minimum volumes <1 mL (Table 4.1). After the completion of this work, we define the 8 ring micromixer and 24 obstruction mixers as our current best options for mRNA-LNP production, both producing similar results.

	Easy Iterations/ Modifiable	**Reproducible mRNA-LNPs**	**Minimum Volume Requirements > 1 mL**	**Flexible Flow Rate Capacity**	**Scalability**	**Costly**
In-House PDMS Mixers	Yes	Yes	No	Yes	Scale Out (Parallelized)	No
Automated Benchtop Mixers	No	Yes	Yes	Yes	Scale Up (Flow Rates)	Yes

Table 4.1. Evaluation of advantages and disadvantages of different types of microfluidic mixers for mRNA-LNPs**.**

To our knowledge, a continuous platform for mRNA-LNP production has yet to be established in literature [128]. We are, however, aware that companies, such as Nutcracker Rx, are developing similar platforms encompassing the mRNA production, purification and encapsulation all within microfluidic channels [129]. Our study contributes to possible strategies for the implementation of downstream processing and formulation steps into a continuous manufacturing platform through the optimization of these unit steps. Furthermore, the results of these studies are not limited to a single application but are more generally applicable to the development of mRNA-LNPs for various pathologies, as well as for siRNA and pDNA-LNP loading. However, as we note during the chapter, in order to increase the resistance of the device to pressures at higher flow rates, alternative manufacturing methods of the microfluidic devices, notably glass based, can be considered as we move further.

In Chapter 3, we evaluated the various possible storage conditions for mRNA-LNPs and screened for formulations of these products which could withstand freeze thaw stresses. We began by identifying appropriate excipients known to preserve lipid enveloped structures at frozen temperatures. We follow this study up by identifying two formulations (F1: 0.5 % P188 and 8% Sucrose, F2: 0.5 % F127 and 8% Mannitol) which aid in the preservation of SM-102 mRNA-LNPs and ALC-0315 mRNA-LNPs at 4°C, -20°C, and -80°C. We highlight the limitation of mRNA stability at room temperature, and the importance of future research on reducing the water contents within the core of LNPs to stabilize the mRNA.

Throughout this chapter, we ensure the inclusion of a surfactant as an excipient in order to allow for future studies employing our protocols to administer particles by inhalation. As this project was established through pandemic preparedness frameworks, we anticipate that this non-invasive delivery method will gain attention with the threat of an incoming respiratory infection pandemic [130]. Inhalable vaccines allow for local administration of the vaccine and stimulation of the mucosal immune response, proving to be a promising immunization strategy [131]. Furthermore, the impact of this work extends beyond biomanufacturing and contributes to reducing vaccine waste and improving sustainability practices when storing mRNA-LNPs. Several researchers have noted that insufficient cold chain capacity has resulted in vaccine wastage which incurs financial consequences [132]. Furthermore, studies have shown the cold supply chain accounts for 69.8%

of energy consumption in the vaccination life cycle [133]. Reducing the requirements for cold chain reliant distribution and storage would represent a critical milestone in the development of mRNA-LNP biologics.

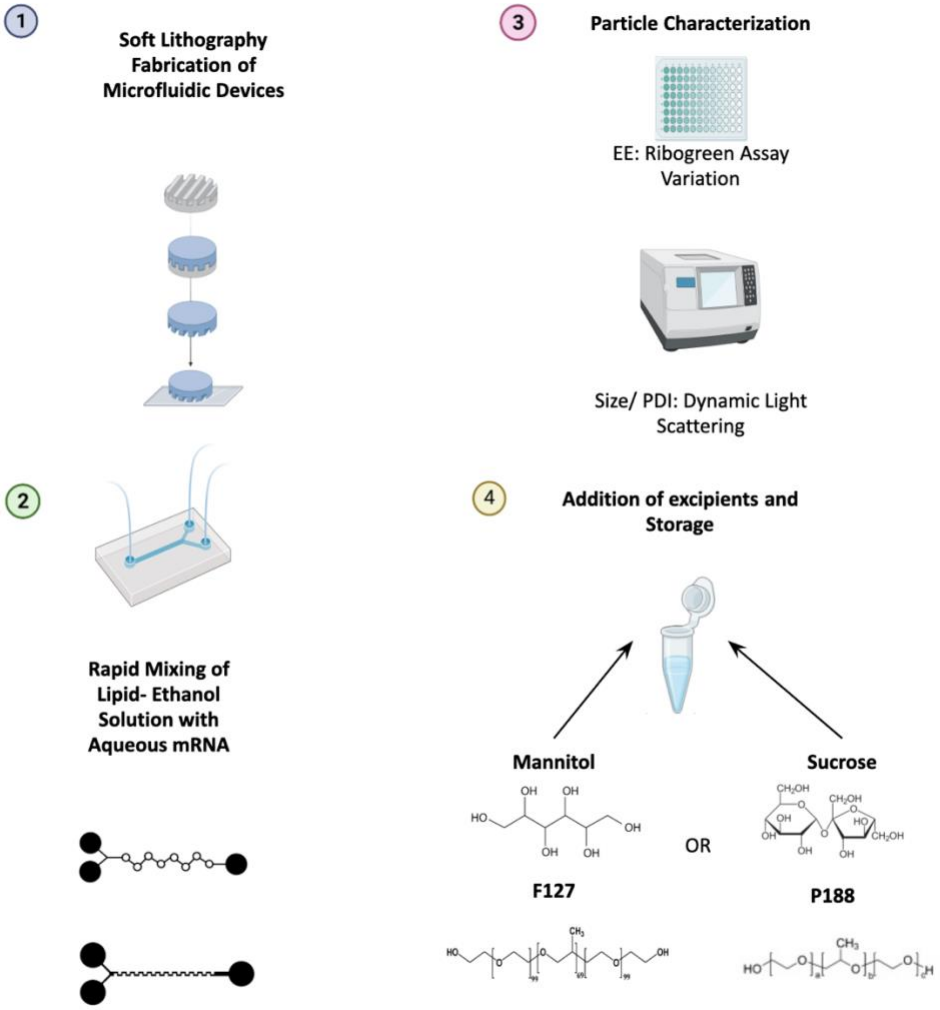


Figure 9.1. Schematic representing the entire process for mRNA loaded LNP manufacturing as optimized throughout the project.

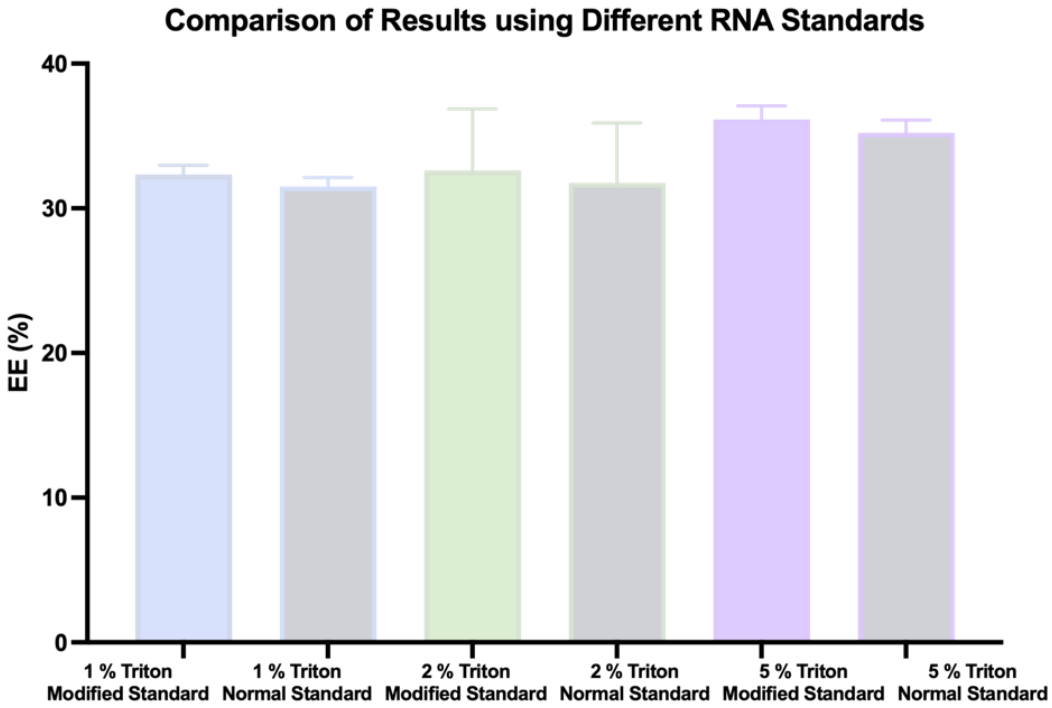
Together, the work from chapter 2 and 3 establishes a working pipeline for the development of these products within our facilities, as represented in Figure 4.1. We also defined and optimized the necessary analytical technologies to maintain and control the quality of these products throughout the manufacturing process, based on the CQAs and manufacturing challenges

identified in chapter 1 (Appendix 1). Additional work to optimize the downstream processing of the particles was performed (Appendix 2) in order to enable this work.

In conclusion, we fulfilled our aims of establishing a flexible manufacturing process for mRNA-LNP development and to improve their storage ability. As the range of applications of mRNA-LNP products expands, and the number of ongoing clinical trials continues to increase, the development of these scalable and cost effective manufacturing processes is critical. Future work involving the development of lyophilized, solid formulations for room temperature storage of LNPs will contribute to the improvement of long term storage and of rapid vaccine manufacturing platforms and will increase vaccine and therapeutic availability in LMIC and vulnerable communities.

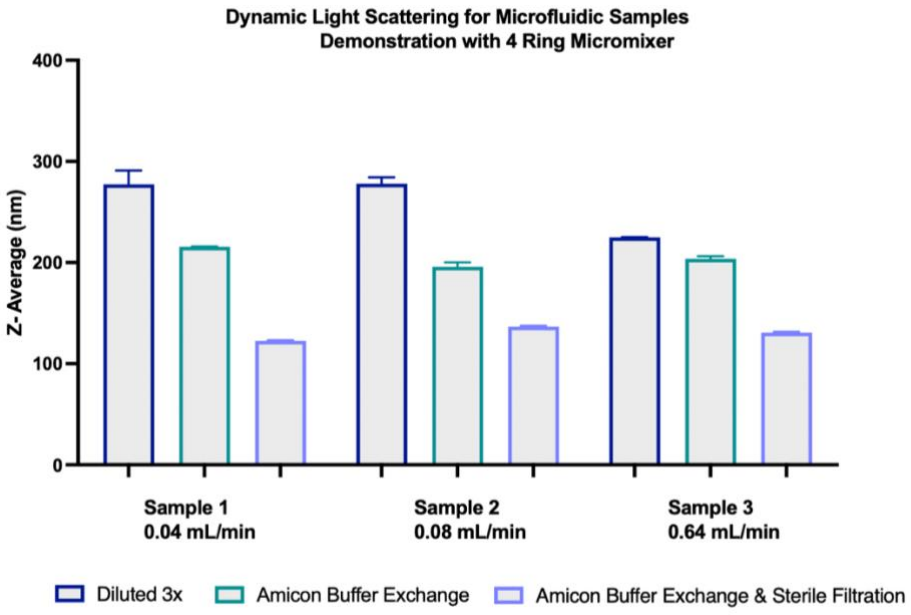
Appendix 1

Figure 10.1. Optimization of Ribogreen Assay Variation for encapsulation efficiency of LNPs. Error Bars represent SD of *n=2* samples.



Due to variations in available Ribogreen protocols for encapsulation efficiency, a comparison triton concentrations and standard curves was conducted in order to establish a protocol for the work. A single sample was assayed under a variety of conditions. The use of 1%, 2% and 5% Triton X-100 were assessed for samples where total mRNA is calculated. Furthermore, a modified standard curve which includes the incubation of Triton X-100 was examined, as well as the normal unmodified standard. We conclude that no significant differences exist between conditions. For simplicity, we choose to use the 2% or 5% Triton concentrations to break open LNP samples with an unmodified standard curve.

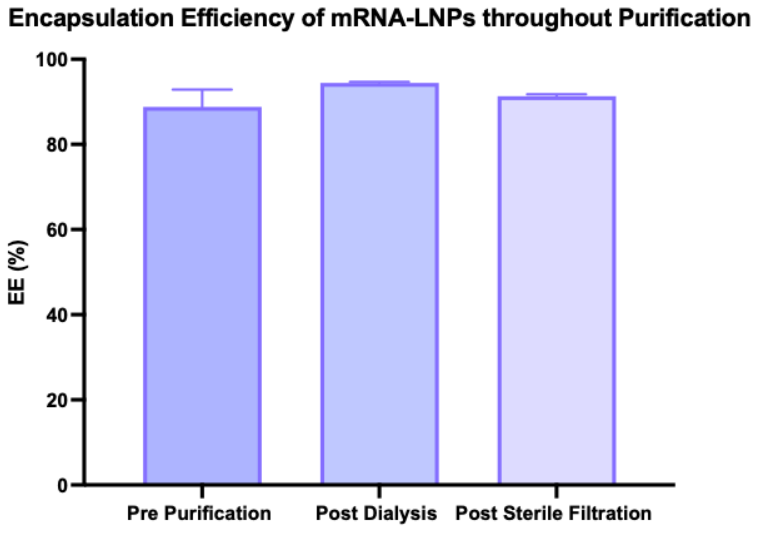
Figure 5.2. Necessary Buffer Exchange and Sterile Filtration for Microfluidic mRNA-LNP samples



After Microfluidic Operation, to remove excess lipids and lipid aggregates prior to DLS analysis, it was recommended to perform a buffer exchange using Amicon MWCO 100kDa tubes and sterile filtering the LNPs produced in order to accurately measure the size of the mRNA-LNPs. We demonstrate here the change in size observed as we perform each step of this process.

Appendix 2

Figure 6.1. Demonstration of maintenance of particles throughout downstream processing of LNP samples



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