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Developing the Next Generation of mRNA Vaccines: Redefining mRNA Delivery Through Rational LNP Manufacturing, Formulation and Targeting Studies

A thesis presented for the degree of

Doctor of Philosophy

from the Institute of Pharmacy and Biomedical Science

at the University of Strathclyde

by

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Declaration of Authenticity

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Signed: Jade Cairney Forrester

Date: 10/04/2026

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Yvonne Perrie. I am incredibly grateful to have been given the opportunity to complete my PhD with you and your continuous support, guidance and encouragement have been instrumental in shaping my professional career.

Additionally, this PhD was supported through funding bodies, namely, the James Weir Foundation and Bio-Techne, without which this project would not have been possible. I would like to take time to thank them for their invaluable input, specifically Alex Moloney at Bio-Techne, Bristol, who allowed me to gain industry insights and offered support and guidance through my end project.

My PhD journey would not have been the same without the amazing lab members I have met throughout. While there are too many to name, Sarah, Rebecca, Catie and Catherine will always hold a special place in my life for the continuous support and lunch time chats over the past few years.

To my closest friends – Cameron, Callum, Emma and Chloe – thank you for always supporting me through every milestone, your unwavering love and welcome distractions helped me get to where I am today. I feel extremely lucky to have you all in my life.

In memory of my dad and to my mum and gran, thank you for everything you have done to help me reach this point. You have been a constant source of love and a guiding light that reminded me what I was always capable of. Everything I do in life is a credit to you.

“Strong in heart and dreams” – Runrig, The Ocean Road

University of Strathclyde

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Abstract

Lipid nanoparticles are now the most clinically advanced lipid-based nucleic acid delivery system – offering a precise and efficient method to intracellular nucleic acid delivery. However, since their clinical success during the COVID-19 pandemic, LNPs have lacked in areas such as fast and simple formulation screening, enhanced endosomal escape and extra-hepatic targeting capabilities. Enhanced screening techniques and optimised manufacturing could help alleviate these issues.

This thesis focused on increasing the availability and accessibility of LNP formulation and manufacture. Initial studies looked at a comparison of formulation screening methods: OFAT, manual, and automated DoE and identified that conclusions drawn from each method were not identical, highlighting how manufacturing can affect the CQAs and hence functionality of LNPs. Following studies then investigated low-cost microfluidic mixers, including pipette mixing as a formulation and high-throughput screening tool, demonstrating the feasibility and efficacy during manufacture. This thesis also identified the need for orthogonal and additional methods to screen RNA encapsulation efficacy in lipid nanoparticles as well as mRNA functionality, investigating HPLC-PDA quantification and ex vivo cellular systems as functional assays. Lastly, final studies were focused on extra-hepatic targeting of lipid nanoparticles, aiming to successfully deliver mRNA to M2-subtype macrophages through post-insertion of a targeting ligand on the outside layer, demonstrating enhanced protein expression for select formulations.

In conclusion, this thesis provided insight into how mRNA vaccine development can be aided by refining current formulation, manufacturing and targeting procedures.

List of Publications

Forrester, J., Davidson, C. G., Blair, M., Donlon, L., McLoughlin, D. M., Obiora, C. R., Stockdale, H., Thomas, B., Nutman, M., Brockbank, S., Rattray, Z., & Perrie, Y. (2025). Low-Cost Microfluidic Mixers: Are They up to the Task? *Pharmaceutics*, 17(5), 566. <https://doi.org/10.3390/pharmaceutics17050566>

Forrester, J., Vaughan, E., Knappett, B. R., & Perrie, Y. (2026). Revisiting the Lipid Ratio in Lipid Nanoparticles: Insights from OFAT and DoE in Formulation and Expression Studies. <https://doi.org/10.1007/s13346-026-02125-6>

Forrester, J., Vennall, G., Wheeler, D., Moloney, A., & Perrie, Y. (2026). First Responders Meet First Class Delivery: LNPs for Macrophage Targeting. (Currently being drafted).

List of Application Notes

RiboDyEE™: A New Cost-Effective Solution for Robust RNA Encapsulation Efficiency Measurement in Lipid Nanoparticles

Jade Forrester, Sarah Lindsay, Rebecca Macfarlane, Alex Moloney and Yvonne Perrie.

Published: Date not confirmed.

In Vivo Evaluation of Standard mRNA-LNP Formulations Produced with TAMARA

Jade Forrester, Claire Council, Audrey Nsamela, Sezen Gul, and Yvonne Perrie.

Published: Date not confirmed.

List of Poster Presentations

Nanomed Europe, June 2023, The University of Liverpool – The Effect of Lipid Composition on the Physicochemical Properties of Lipid Nanoparticles.

18th Liposome Research Days, June 2024, The University of Strathclyde – Can a Mixture Design of Experiments (DoE) Approach Produce a Sufficient Predictive Model for Lipid Nanoparticle Formulation Optimisation.

18th Liposome Research Days, June 2024, The University of Strathclyde – Optimising Manufacturing Parameters for mRNA-LNPs Using the Nova™ Benchtop.

Controlled Release Society, July 2024, Bologna Conference Centre – Understanding the Effect of Lipid Composition on the Physicochemical Properties and Transfection Efficacy of Lipid Nanoparticles.

RNA Vaccines and Therapeutics Conference, October 2024, The Royal College of Physicians – Reliability of Low-Cost Microfluidic Mixers: Are They Up to the Task?

21st Annual bioProcess UK Conference, November 2024, Liverpool - Reliability of Low-Cost Microfluidic Mixers: Are They Up to the Task? (**bioProcess Travel Grant awarded**).

APS PharmSci 2025, September 2025, Cardiff – Revisiting the Lipid Ratio in Lipid Nanoparticles: Insights from a DoE in Formulation and Expression Studies. (**Awarded Best Poster Prize**).

PGR Research Day, October 2025, The University of Strathclyde - Revisiting the Lipid Ratio in Lipid Nanoparticles: Insights from a DoE in Formulation and Expression Studies.

Controlled Release Society, July 2026, Portugal – First Responders Meet First Class Delivery – LNPs for Macrophage Targeting (Upcoming).

List of Oral Presentations

21st Annual bioProcess UK Conference, November 2024, Liverpool - Reliability of Low-Cost Microfluidic Mixers: Are They Up to the Task? (**bioProcess Travel Grant awarded**).

PGR Research Day, October 2025, The University of Strathclyde - Revisiting the Lipid Ratio in Lipid Nanoparticles: Insights from a DoE in Formulation and Expression Studies.

Lunch & learn, January 2026, Bio-Techne, Bristol – First Responders Meet First Class Delivery: LNPs for Macrophage Targeting.

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Abbreviations

Full Descriptor	Abbreviation
Lipid Nanoparticles	LNPs
deoxyribonucleic acid	DNA
ribonucleic acid	RNA
messenger ribonucleic acid	mRNA
Intravenous	I.V.
1,2-di-O-octadecenyl-3-trimethylammonium propane	DOTMA
Hydrogenated phosphatidylcholine	HSPC
Cholesterol	Chol
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]	DSPE-PEG2000
Small Interfering Ribonucleic Acid	siRNA
Hereditary transthyretin amyloidosis	hATTR
Critical Micelle Concentration	CMC
Nanometres	nm
1,2,-distearoyl-sn-glycero-3-phosphocholine	DSPC
Dioleoylphosphatidylethanolamine	DOPE
1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol)	C12-200
1,2,-dimyristoyl-rac-glycerol-3-methoxy-polyethylene glycol-2000	DMG-PEG2000
Distearoyl-rac-glycerol-PEG2000	DSG-PEG2000
Acid Dissociation Constant	pKa
Antisense oligonucleotides	ASO
Critical Quality Attributes	CQAs
Design of Experiments	DoE
Self-amplifying Ribonucleic acid	saRNA
Critical Process Parameters	CPPs
Nitrogen-to-Phosphate Ratio	N/P Ratio
Potential of Hydrogen	pH
Phosphate Buffered Saline	PBS
Polydispersity Index	PDI
(6Z, 9Z, 28Z, 31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate	DLin-MC3-DMA
Enhanced Green Fluorescent Protein	eGFP

Tris Buffered Saline	TBS
HEPES Buffered Saline	HBS
N1-methylpseudouridine	1MpU
Non-Human Primate	NHP
Flow Rate Ratio	FRR
Total Flow Rate	TFR
Small Unilamellar Vesicles	SUVs
Dynamic Light Scattering	DLS
The European Medicines Agency	EMA
Food and Drug Administration	FDA
Nanoparticle Tracking Analysis	NTA
Flow Field Fractionation	FFF
Multi-Angle Light Scattering	MALS
Ultraviolet	UV
Electrophoretic Light Scattering	ELS
High Performance Liquid Chromatography	HPLC
Cryogenic Transmission Electron Microscopy	cryoTEM
Nuclear Magnetic Resonance	NMR
Clathrin-Mediated Endocytosis	CME
Apolipoprotein E	ApoE
Low-Density Lipoprotein Receptor	LDL-R
Selective Organ-Targeting LNPs	SORT LNPs
Intramuscular	I.M.
Respiratory Syncytial Virus	RSV
Heptadecan-9-yl 8-[(2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino]-octanoate	SM-102
(4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl)bis(2-hexyldecanoate	ALC-0315
Methoxypolyethyleneglycoloxy (2000)-N, N-ditetradecylacetamide	ALC-0159
Tangential Flow Filtration	TFF
1,2-dioleoyl-3-trimethylammonium-propane	DOTAP
Diethyl Pyrocarbonate	DEPC
Polyadenylic Acid	Poly A
Human Embryonic Kidney - 293	HEK-293
Staggered Herringbone Mixer	SHM
Firefly Luciferase	Fluc
Tris EDTA	TE
Carbon Dioxide	CO ₂

T-junction Mixer	TM
Confined Impingement Jet Mixer	IJM
AXF-Mini Mixer	AXFM
Green Fluorescent Protein	GFP
1,2-dioleoyloxy-3-(dimethylamino) propane	DODAP
1,1'Dioctadecyl-3,3,3',3'-tetramethylinodotricarbocyanine iodide	DiR
1,1'-Dioctadecyl-3,3,3',3'-Tetramethylinodcarbocyanine perchlorate	DiIC
Cyanine 5	Cy5
Nucleic Acid Therapeutics	NAT
Nanoparticles	NPs
Manual Design of Experiments	mDoE
Automated Design of Experiments	aDoE
Fetal Bovine Serum	FBS
Minimal Essential Media	MEM
Cell-Free Protein Expression	CFPE
High Performance Liquid Chromatography – Evaporated Light Scattering Detector	HPLC-ELSD
Triethylamine Acetate	TEAA
Triethylammonium	TEA
Standard Deviation	SD
Standard Error of the Mean	SEM
<i>In vitro In vivo</i> Correlation	IVIVC
Quality-by-Design	QBD
Pipette Mixing	PM
Toroidal Mixer	TM
Rabbit Reticulocyte Lysate	RRL
Frit-Inlet Asymmetric Flow Field-Flow Fractionation	FI-AF4
Green Lantern	GrL
Radius of Gyration	Rg
Hydrodynamic Radius	Rh
Full Width Half Max	FWHM
Dibenzocyclooctyne	DBCO
Mannose Receptor	CD206
Strain-Promoted Alkyne-Azide Cycloaddition	SPAAC
Interleukin-4	IL-4
Lipopolysaccharide	LPS

Interferon Gamma	IFN- γ
Interleukin-13	IL-13
Toll Like Receptor	TLR
Interleukin-6	IL-6
Reactive Oxygen Species	ROS
Tumour-Associated Macrophages	TAMs
Platelet-derived Growth Factor	PDGF
Vascular Endothelial Growth Factor	VEGF
Tumour Immune Cell Targeting Chimeras	TICTACs
Dulbecco's Minimal Essential Media	DMEM
Ion-Pair Reverse Phase Liquid Chromatography	IP-HPLC
Red Fluorescent Protein	RFP
4',6-diamidino-2-phenylindole	DAPI
Limit of Detection	LOD
Limit of Quantification	LOQ
Cell Total Corrected Fluorescence	CTCF
Potassium Hydroxide	KOH
Adenosine Triphosphate	ATP
Guanosine Triphosphate	GTP
Standard Operating Procedures	SOPs
Propidium Iodide	PI
1,2-dimyristoyl-racglycero-3-(azidopolyethylene glycol-2000)	DMG-PEG2000-azide
1,2-distearoyl-sn—glycero-3-phosphoethanolamine- <i>N</i> -[carbonyl azido(polyethyleneglycol)-2000]	DSPE-PEG2000-azide

Chapter 1: General

Introduction

Type of Publication	☐ Published article ☐ Accepted for publication ☐ Submitted for publication ☐ Part-Drafted for publication/application notes ☒ Not applicable
Citation Details (only applicable if published)	N/A
Chapter Inclusion Details	☐ This chapter is a word-for-word reproduction of the published article ☐ This chapter contains minor revisions of a drafted application note. ☐ The majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	N/A

1.1 The Roadmap to Lipid Nanoparticles

The scientific development of lipid nanoparticle-based nucleic acid therapeutics is the result of decades of complex research, with the key stages identified in Figure 1-1. The 1950s marked the beginning, when Francis Crick first proposed the unidirectional flow of genetic information (DNA > mRNA > protein) and the linear sequence of genetic information through the Central Dogma and Sequence Hypothesis [2-4]. This discovery was revolutionary for nucleic acid-based therapeutics as it highlighted the potential benefit of introducing genetic material into cells. Shortly after this, Alec Bangham identified that lecithin and cholesterol mixtures were forming lamellae-like structures through negative staining of phospholipids – these structures were termed liposomes and marked the beginning of lipid-based delivery systems [5-7]. While these liposome systems showed considerable promise, they were limited by manufacturing challenges, particularly the need for size control. Over the following decade, key advances addressed these issues, notably the work of Batzri et al., who developed methods to produce single-layer liposomes without requiring additional size control steps [8]. Furthermore, Olson et al. demonstrated that liposome size could be controlled by extrusion through polycarbonate membranes [9]. The true therapeutic power of liposomes then became apparent in 1983, when Nicolau et al. and Soriano et al. demonstrated successful incorporation of the preproinsulin I gene in rats following I.V. administration of liposomes composed of egg yolk phosphatidylcholine, cholesterol, and ox brain phosphatidylserine [10, 11]. Simultaneously, researchers were establishing methods that allowed for successful *in vitro* transcription of functional RNAs [12, 13]. While early studies demonstrated the feasibility of nucleic acid delivery, with DNA and RNA successfully incorporated and delivered via both intraperitoneal and intramuscular injection in rats and mice [14, 15], liposomes were concurrently emerging as a versatile delivery platform, particularly through the use of pH gradient-driven drug loading [16, 17]. In the 1980s, DNA/RNA was

successfully transfection in cell culture using a small unilamellar liposome structure consisting of the cationic lipid DOTMA [18]. These cationic lipids then dominated a large portion of liposome formulations due to their ability to create lipid-nucleic acid complexes. A key milestone occurred in 1995 when the first liposomal-based product, Doxil® (also known as Caelyx® in Europe), gained regulatory approval. Doxil® is a PEGylated liposomal formulation that promotes high drug entrapment via the formation of a rigid bilayer [19, 20]. The liposomal formulation consists of three lipids: hydrogenated phosphatidylcholine (HSPC), cholesterol (Chol) and DSPE-PEG200 at a w/w ratio of 3:1:1, which entrapped the chemotherapeutic drug doxorubicin in the form of doxorubicin-sulphate crystals [19]. The FDA approval of Doxil® was primarily focused on minimising the adverse effects experienced by patients treated with doxorubicin and has been widely employed and successful [19]. During the 1990s, another important line of research was the discovery that PEGylated lipids could control the fusogenic activity of liposomes, which in turn was driven by the amount of PEGylated lipids incorporated [21]. While cationic lipids were the key players in early liposome research, their inherent toxicity soon became apparent, prompting the search for alternatives. Researchers developed ionisable lipids that are protonated at lower pH, allowing them to complex with nucleic acids, but become neutral at acidic pH, thereby reducing the toxicity associated with cationic lipids [22]. These discoveries led to a new nucleic acid-based delivery system, termed lipid nanoparticles, consisting of a four-lipid structure that will be further explored in the following sections. Researchers began to highlight the successful delivery of siRNA through the injection of these lipid nanoparticles [23, 24]. This led to clinical trials of an siRNA-LNP formulation aimed at treating hereditary transthyretin (hATTR) amyloidosis [25], which was subsequently approved in 2018 [25]. Simultaneously, mRNA delivery kinetics were also being explored [26], and the COVID-19 pandemic in 2021 offered a unique opportunity for pharmaceutical companies to advance mRNA-LNP-based research, resulting in

two approved vaccines [22, 27]. This brings us to today, when several nucleic acid-LNP vaccines have been approved, and many more are in clinical trials.

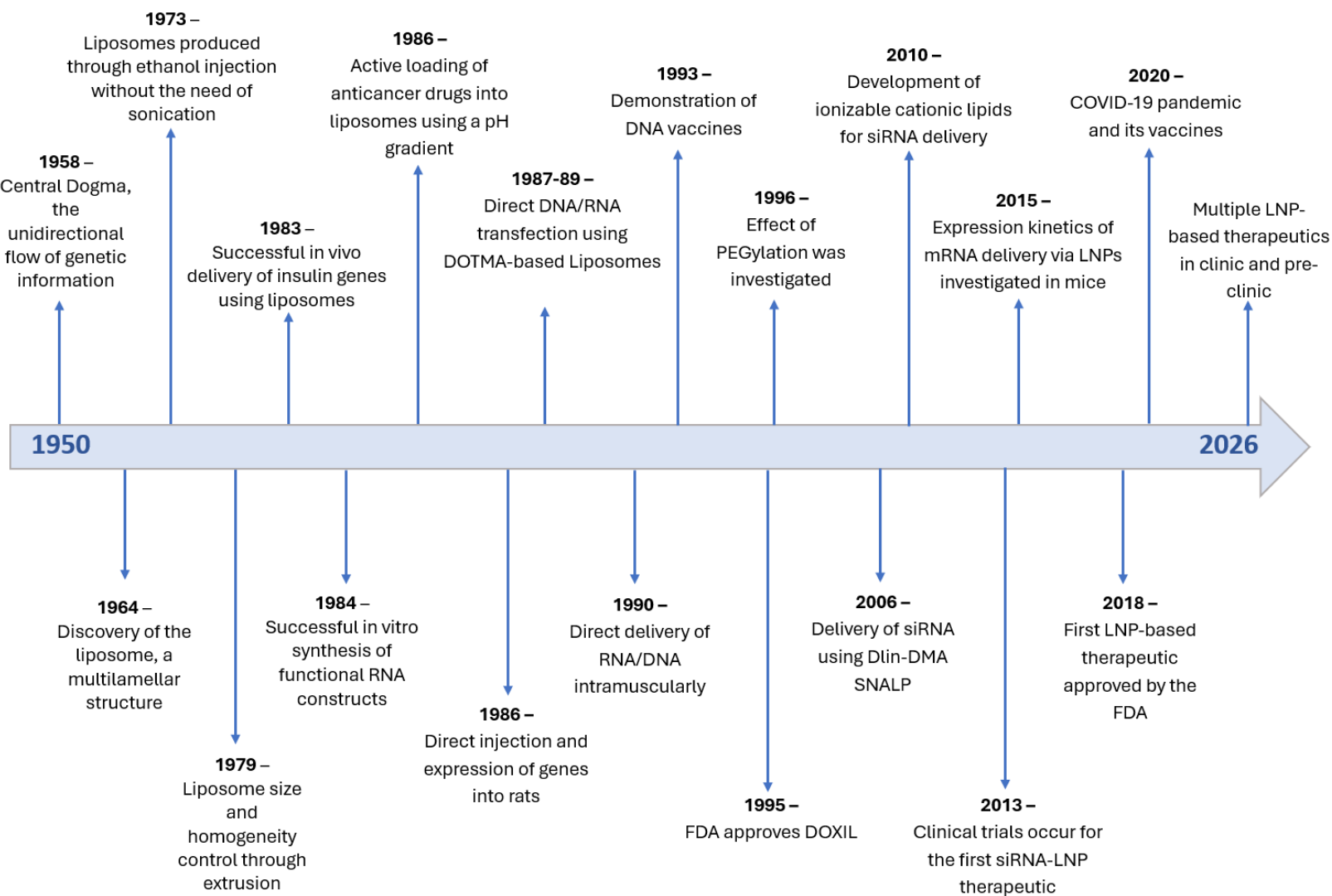


Figure 1-1 Timeline of Development for Lipid-Based Delivery Systems. This timeline highlights only a number of key articles or scientific discoveries that aided in the development of modern-day mRNA-LNP formulations. Graphic was developed in Microsoft Word.

1.2 Exploring Alternative Nanoparticles

This thesis will primarily focus on lipid nanoparticles; however, it is important to discuss that several other nanoparticle formulations are being investigated to address therapeutic challenges, and some of these will be discussed here. Nanomaterials are a broad category, and general classification is dependent upon how many of their dimensions are within the nanoscale, giving rise to zero-dimensional (0-D), one-dimensional (1-D), two-dimensional (2-D) and three-dimensional (3-D) [28]. Nanoparticles fall into the 0-D category due to all their materials residing on the nanoscale [28]. From here, nanoparticles are further classified depending on whether they are organic, inorganic, or carbon-based [28, 29].

1.2.1 Organic Nanoparticles

Organic nanoparticles are described as consisting of organic material, such as polymers and lipids, and generally possess non-toxic and biodegradable qualities [28]. Furthermore, these nanoparticles can be sensitive to thermal and magnetic signals, making them particularly interesting for drug delivery [30]. Some examples of organic nanoparticles are detailed here.

1.2.1.1 Liposomes versus Lipid Nanoparticles

The two most common organic nanoparticles are liposomes and lipid nanoparticles; while lipid nanoparticles evolved from liposomes, there are key differences between them. Liposomes are generally considered spherical structures consisting of an outer lipid bilayer encapsulating a liquid core, offering the unique ability to encapsulate both lipophilic and hydrophilic drugs [31]. Whereas LNPs consist of a morphology which can be described through three sections, the outer shell typically consisting of phospholipids, helper lipids and PEGylated lipids, while the inner shell consists mainly of phospholipids and ionisable lipids encapsulating a nucleic acid payload core [32]. The key structural differences of liposomes and LNPs are illustrated in Figure 1-2. LNPs are generally considered optimal for nucleic acid delivery due to a high degree of

control over nanoparticle characteristics and the ability to encapsulate virtually any nucleic acid payload or construct with minimal formulation optimisation [33].

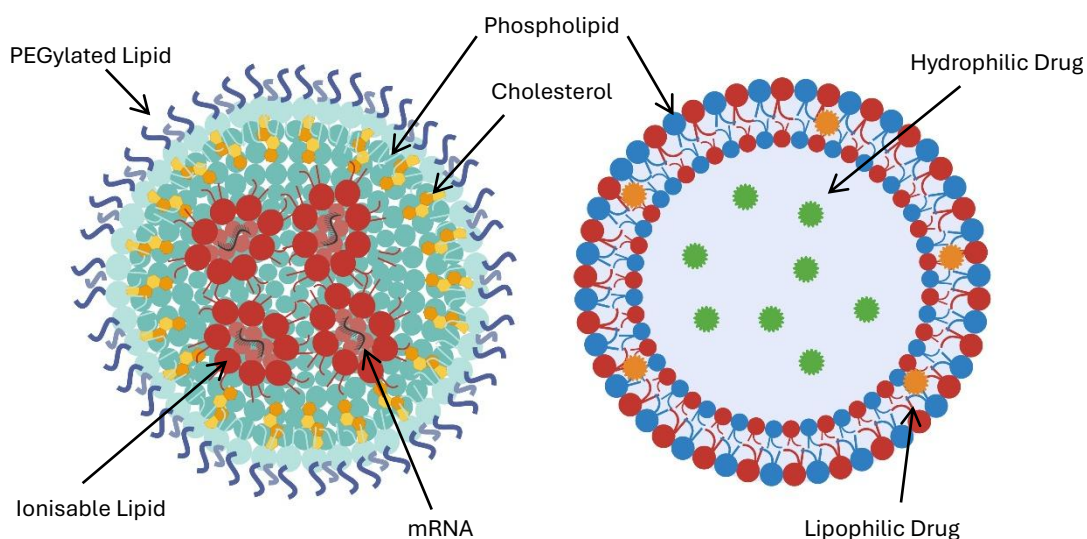


Figure 1-2 Describes the Structure of a Standard Liposome and Lipid Nanoparticle. LNPs exhibited a four-lipid mixture of PEGylated lipids, phospholipids, cholesterol and ionizable lipid encapsulating mRNA. While liposomes consist of a phospholipid bilayer surrounding an aqueous core with possible lipophilic drugs shown in orange and possible hydrophilic drugs shown in green. Graphic was generated on Bio-Render.

1.2.1.2 Micelles

Micelles are described as single polymer chains which assemble into polymeric micelles, resulting in a spherical inner core which is hydrophobic and a hydrophilic outer shell [34]. Meewan et al. demonstrated that conjugating PEG lipids to micelles resulted in a stealth formulation [35]. These nanoparticles have been shown to increase the bioavailability of lipophilic drugs; however, they offer limited drug loading capacities, and if the critical micelle concentration (CMC) is not reached, the structures can disassemble prematurely in the blood [36].

1.2.1.3 Polymeric Nanoparticles

Polymeric nanoparticles are generally solid colloidal structures composed of repeating monomers [29] that can be classified as nanosphere or nanocapsule systems, depending upon the internal structure of the polymeric nanoparticles and whether the drug is retained or dissolved within these structures [37]. A major advantage of these systems is their lower toxicity profiles [29].

1.2.1.4 Dendrimers

Dendrimers are termed as symmetric globular branched structures that, with small sizes, are 1 to 15 nm [38]. Dendrimers are generally employed to aid in the bioavailability of drugs and have a wide range of medical and non-medical uses [38]. Advantages of these systems include their monodispersed nature, small sizes and ability to modulate pharmacokinetics [38, 39].

1.2.2 Inorganic Nanoparticles

Inorganic nanoparticles are classified as any nanoparticle system that is not composed of organic or carbon materials [28, 30]. They are generally non-toxic, biodegradable and exist as a hydrophilic material [30]. Brief examples are described below.

1.2.2.1 Metallic Nanoparticles

Metal nanoparticles are particles that are entirely composed of metals such as gold, silver, palladium and iron [40]. These nanoparticles are gaining significant interest, especially in anti-cancer systems, due to their strong optical properties, which can allow for photo-thermal activity as well as imaging applications [41]. Alongside this, metal nanoparticles also exhibit ease of manufacture due to their simple structures and flexible functionalisation [42].

1.2.2.2 Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles are composed of ordered nanosized silica, resulting in a structure with pores that typically range between 2 and 50 nm and that can increase surface area and allow encapsulation of drugs [43, 44]. These structures offer advantages over other nanoparticles as they can allow drugs to remain in amorphous states, improving dissolution [44].

1.2.2.3 Quantum Dots

Quantum dots are nanocrystal structures that can have a cadmium, indium, or carbon core, encapsulated by chalcogenides [45]. These particles generally

exhibited spherical structures which are between 2 and 20 nm [46]. Fluorescent imaging and labelling are the key uses due to their fluorescent stability and fluorescence lifetime [46].

1.2.3 Carbon-Based Nanoparticles

Carbon-based nanoparticles can be described as nanoparticles that are solely comprised of carbon atoms [28]. These nanoparticles are particularly advantageous due to carbon being readily available, as well as having the ability to form a multitude of bonds with other elements [47]. Carbon-based nanoparticles, including fullerenes, are classed as an allotrope of carbon that exists as a cage structure of sp^2 carbons [48]. These nanoparticles are beneficial as they can react differently to different stimuli, as well as being non-immunogenic and low-cost [48]. Furthermore, carbon-based nanoparticles can include nanotubes, which can exist as single-walled or multi-walled, and researchers have demonstrated that these can be conjugated with molecules such as aptamers to allow for receptor-independent cellular translocation [49]

1.3 Lipid Nanoparticle Structure

LNP efficacy and functionality are highly linked to their structure. Generally, they exhibited a spherical structure consisting of a four-lipid mixture composed of phospholipids, cholesterol, ionisable lipids and PEGylated lipids, which encapsulate a nucleic acid core [22, 32, 33]. Each component plays a specific role in the structure, characterisation, and functionality, which will be explored in the following sections.

1.3.1 Forming the Lipid Shell – Phospholipids and Cholesterol

Phospholipids typically make up 10% of the complete molar ratio of LNP formulations [50]. They are utilised in LNPs to enhance mRNA encapsulation through stabilisation of the LNP structure, as well as promote efficient mRNA delivery through destabilisation of the endosomal membrane [50-52]. The

structure of phospholipids can be described as a phosphate group with two conjugated hydrophobic tails [53]. In all approved LNP vaccines, the phospholipid is DSPC, which consists of two saturated 18-carbon tails conjugated to a phosphatidylcholine headgroup [50]. While the high transition temperature of saturated phospholipids such as DSPC is beneficial, as it can increase LNP stability, it also poses a risk of maintaining a too-rigid structure, which can affect endosomal escape [54]. This is a plausible reason why unsaturated lipids such as DOPE can increase protein expression [55]. The lower transition temperature of DOPE allows the lipid layer to be in a more fluid form, which could facilitate membrane fusion and enhance mRNA delivery [50]. Despite the clinical success of DSPC-containing LNPs, researchers have strived to enhance these properties. For example, Gan et al. screened a library of >100 phospholipids containing adamantyl groups and identified a formulation that enhanced extrahepatic mRNA delivery [56]. Furthermore, DOPE has been shown to favour liver accumulation, whereas DSPC favours splenic accumulation when used to prepare C12-200-LNPs injected intravenously into mice [57]. This highlights how phospholipid structure can influence efficiency, functionalisation and biodistribution of LNP formulations.

Cholesterol typically comprises 38.5% of a standard LNP formulation and exhibits a planar rigid structure, which can enhance LNP stability through 'filling the gaps' in the bilayer [51, 52]. Inclusion of sterols also enhanced biocompatibility due to being a naturally occurring material that is available in bulk extracts [50]. Kawaguchi et al. highlighted how decreasing the percentage of cholesterol in LNP formulations resulted in a lower protein expression level [58]. This highlights how cholesterol can enhance membrane fusion and hence payload release. Furthermore, studies highlight discrepancies, demonstrating that natural derivatives of cholesterol (such as β -sitosterol) can enhance mRNA delivery and transfection [59, 60], indicating that each lipid may demonstrate varied functionality depending on the other lipids within the formulation. Patel et

al. also demonstrated that both polarity and sterol ring flexibility are key determinants of transfection efficiency, with group II cholesterol analogues exhibiting higher transfection efficiency [61].

1.3.2 Coating the Shell – PEGylation

PEGylated lipids comprise the smallest molar ratio of LNPs, ~1.5% and enhance bioavailability through prevention of protein binding and offering steric hindrance, which can control particle size [22, 53]. PEGylated lipids are usually composed of polyethylene glycol molecules attached to alkyl chains, and during manufacture, the alkyl chains extend away from the core, promoting self-assembly [62]. The transfection efficiency of LNPs is dependent upon dissociation of the PEG lipid from the surface, and the length of the PEG alkyl chain has been identified as a key contributor to dissociation rates, with shorter tails having higher desorption [62]. It should also be noted that PEGylated lipids carry a low risk of anti-PEG antibodies, as IgM can rapidly clear PEG, thereby decreasing transfection efficacy and introducing the possibility of adverse effects [63]. The most abundant PEGylated lipids in LNP research are DMG-PEG₂₀₀₀, and Borah et al. highlighted how DMG-based LNPs offered enhanced transfection efficacy over DSG-based LNPs [64]. Early research also established PEGylation as a key determinant of LNP size; for example, Kulkarni et al. demonstrated that increasing PEG content from 0.5–2.5% resulted in a decrease in particle size [65]. Furthermore, modifications to PEGylated lipids that alter charge could also increase protein expression, highlighting the flexibility of these components in LNP systems [66, 67].

1.3.3 Filling the Core – Ionisable Lipids

Ionisable lipids comprise the majority of the LNP formulation – approximately 50% - and are key drivers responsible for RNA protection and endosomal escape [22, 68]. The structure of ionisable lipids is usually denoted by three individual parts [53]. Ionisable lipids exhibit a unique function: they are protonated at lower

pH, such as those encountered during manufacture, allowing complexation of the payload and subsequent release in the cytosol [22, 53]. The headgroup of ionisable lipids is vital to LNP functionality, as it is where the amine resides, and this amine drives the complexing with the nucleic acid payload through electrostatic interaction with the phosphates in the nucleic acid backbone [69]. Furthermore, the headgroup is a key determinant of lipid charge and polarity, which can affect overall efficacy [70]. The next structural component of ionisable lipids is the linker group. These linker groups are often vital for rapid metabolism and easy elimination [69], and while a range of linkers is available, esters are commonly preferred due to their biodegradable nature [53]. Lastly, the ionisable lipids generally possess a tail that is branched, saturated, unsaturated, or mixed [53]. The structure of the ionizable lipid can drive the overall pK_a of the LNP which in turn can determine in vivo efficacy [71]. The acid dissociation constant (pK_a) value is described as 'value equal to pH when an acidic functional group is in dynamic equilibrium with its protonated and deprotonated forms under constant pressure and temperature' [72], and previous research has identified an ideal pK_a to be optimal between 6.2 and 6.5, and different pK_a can affect biodistribution [71, 73]. The electrostatic interactions can result in stabilisation or destabilisation of the amine headgroup protonation state while the hydrophobic tails can alter the local environment surrounding the headgroup which can in turn modulate pK_a shifts [74, 75]. Finally, the overall structural shape of the ionisable lipid is identified as a key design point, with conical shapes thought to disrupt membranes more efficiently, leading to increased payload release [76, 77].

1.3.4 It is What is Inside that Counts – Nucleic Acid Payloads

Nucleic acid therapeutics are among the most promising pharmaceutical agents due to their ability to produce, silence, or enhance the expression of numerous proteins. However, with a delivery vehicle, there are concerns around insufficient protein expression due to mRNA being highly unstable [78], and its large negative nature makes it difficult for it to pass through the cell membrane [79]. This

highlights the key reasons why LNPs are vital to successful nucleic acid delivery. Arguably, the most vital component of LNPs is the nucleic acid therapeutic itself. The most prominent nucleic acid payload in LNP research was small interfering RNA (siRNA), with recent years shifting focus to messenger RNA (mRNA) [80]. Despite this, LNPs are capable of encapsulating virtually any nucleic acid payload, with RNA normally preferred over DNA, as the need for delivery into the nucleus is avoided [81]. Nucleic acid strands typically undergo chemical modification to enhance transcription and stability; this will be explored more in the following sections. Additionally, it has been shown that smaller nucleic acid constructs such as antisense oligonucleotides (ASO) and siRNAs can confer lower encapsulation efficiencies and altered critical quality attributes (CQAs) [80]. Furthermore, discrepancies have been highlighted in research, with some articles highlighting that different nucleic acids follow the same trends with the same formulations and DoE studies showing that formulation optimisation varies depending on what payload is employed [82-84]. Self-amplifying RNA is also gaining interest as a therapeutic, as it would negate the need for repeated dosing and allow for overall doses to be lowered; nevertheless, it has proven difficult to encapsulate compared to mRNA [85]. However, cationic lipid LNPs have been suggested as an alternative delivery option over ionizable lipid LNPs [86]. Overall, this highlights that nucleic-acid LNPs are flexible, but it begins to question the flexibility of standard formulations.

1.4 Critical Process Parameters of Lipid Nanoparticles

The efficacy and functionalisation of LNPs are highly dependent upon the conditions in which they are formulated – also termed critical process parameters (CPPs). CPPs directly influence the self-assembly process and hence are key drivers of LNP size, heterogeneity, charge, encapsulation efficacy and therefore efficacy. This section will briefly explore some of the key CPPs utilised during LNP formulation and manufacture.

1.4.1 The Nitrogen-to-Phosphate Ratio

The Nitrogen-to-Phosphate ratio (N/P ratio) is the ratio of amines group from the ionisable lipid to the phosphate groups from the nucleic acid payload [87]. This is a vital feature in the determination of LNP charge as well as encapsulation efficiency [87]. The current approved LNP-vaccines and therapeutics utilise an N/P ratio that is between 3 and 6 [88] and researchers often utilise this difference during studies [89]. It is suggested that lower N/P ratios inhibit encapsulation efficacy due to insufficient binding potential; however, on the contrary, increased cationic lipid availability has also been shown to hinder encapsulation efficiency, illustrating how a fine balance is required [87, 90]. Li et al. completed a study showing the effect of payload distribution in mRNA-LNPs as a result of the N/P ratio [91]. They highlighted that at low pH, there was a strong positive correlation between empty LNPs and the N/P ratio, resulting in fusion of empty LNPs with mRNA-LNPs and at high pH, high N/P LNPs exhibited increased helper lipid concentrations and high overall LNP concentrations [91]. This illustrates the key role of the N/P ratio, and care should be taken during formulation optimisation.

1.4.2 The Effect of Buffer Choice

Another key parameter up for debate is buffer choice. During manufacture, LNPs required an acidic buffer to enable protonation of the ionisable lipid, thereby facilitating payload complexation [92]. These are commonly citrate-based buffers due to their ability to prevent degradation [93]. Following manufacture, LNPs will be purified into a neutral buffer such as PBS and Tris. This can introduce changes in the structure of LNPs that must be understood. Regarding the internal citrate buffer, increases in molarity have been shown to decrease cellular uptake and, hence, expression, but have no significant effect on particle size, PDI, or encapsulation efficiencies [92]. Furthermore, Carucci et al. demonstrated, using a DLin-MC3-DMA-based LNP formulation, that LNPs manufactured with citrate buffer exhibited higher eGFP expression than those manufactured with

phosphate or acetate buffer, they suggested that citrate buffer stabilised the hexagonal phase during shifts to a higher pH which helped to enable the pH response core in turn enhancing endosomal escape [94]. In addition, post-purification buffers have been shown to affect LNP efficacy. Henderson et al. highlight that LNPs purified in TBS, PBS, and HBS showed no initial differences in CQAs, but after storage, PBS- and HBS-purified LNPs exhibited large size changes [95] as well as, demonstrating that LNPs purified in TBS exhibited increased in vivo expression theorising that this could be due to reduced membrane thickness leading to increased ApoE-mediated uptake. Shkodra et al., also highlighted that LNPs purified in Tris-sucrose exhibited higher in vivo activity compared to PBS LNPs [96]. This could be explained by changes in morphology with Meulewaeter et al., demonstrating that fresh Tris LNPs had an amorphous core structure compared to a blebbed structure seen in PBS LNPs[97]. However, blebbed structures have previously been highlighted as key drivers of improved transfection efficacy [98]. Finally, when it comes to long-term storage, the choice of buffer becomes a significant challenge, especially for temperature-dependent storage, and will require optimisation [99, 100].

1.4.3 mRNA Structure and Modification

While LNP-vaccines rely on synthetic mRNA strands for therapeutic effect, unmodified mRNA is often depicted to result in decreased mRNA expression compared to modified [101]. The majority of approved LNP-vaccines harbouring mRNA also contain N1-methylpseudouridine (1MpU) in place of uridine, and this is thought to enhance mRNA stability and translation [102, 103]. The structures of 1MpU and uridine, as well as general mRNA strands, are highlighted in Figure 1-3. Bernard et al. conducted a study comparing the expression of modified and unmodified mRNA strands from three LNP formulations in both mice and monkey models [103]. They identified that the modification had a significant impact on functional antibody titres in both species, as well as the induction of cytokines and chemokines [103]. However, sequence-optimised unmodified mRNA has

also been shown to successfully induce protective antibody titres against rabies and influenza in a non-human primate (NHP) species [104]. These studies highlight the complexity of payload optimisation, and structural needs will depend highly on the use of the therapeutic or vaccine.

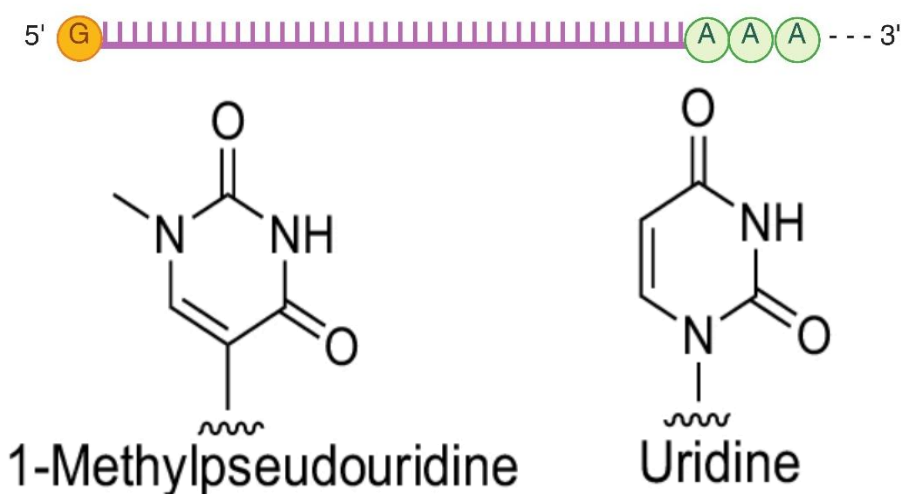


Figure 1-3 Simplified Structure of mRNA Alongside Comparisons of the Structure of Modified and Unmodified base pairs. Image was prepared on Bio-Render as well as being adapted from [1].

1.4.4 Flow Rate Ratios (FRR) and Total Flow Rates (TFRs)

Following the formulation CPPs, the manufacture of LNP vaccines also involves several variables that can modulate the CQAs of LNPs. Two vital parameters in LNP manufacture are the ratio of the aqueous to lipid phases (FRR) and the mixing speed (TFR). The importance of FRR has been well highlighted across the literature. For example, McMillan et al. demonstrated that FFRs of 1.5:1 produced larger LNPs that were less effective compared to a FRR of 3:1 [105]. Furthermore, Roces et al. also demonstrated that increasing FRR from 1:1 to 3:1 resulted in a decrease of around 70 nm in LNP size [106]. The effect is not formulation-dependent, as the same control of size is also evident in lipid-polymer

nanoparticles [107], temperature-sensitive liposomes [108] and standard liposome formulations [109]. On the other hand, TFR is also a method of size regulation with faster speeds equalling smaller sizes [106]. Lower TFRs leading to larger sizes can be explained through the process of Ostwald ripening, which states that changes in solubility of nanoparticles will result in smaller particles redissolving and allow larger particles to grow [110, 111]. These CPPs will be explored more in Chapter 2.

1.4.5 Microfluidic Phase Mixing Methods

Previous manufacture of lipid-based delivery systems was done using techniques which often required downstream processing techniques to control size or encapsulate drugs [112]. However, advances in manufacturing have led researchers to favour microfluidic mixers for LNP manufacture. Microfluidic mixing is the process by which an ethanol lipid-containing phase and an aqueous nucleic acid-containing phase are mixed under laminar or turbulent flow, resulting in small unilamellar vesicles (SUVs). Use of microfluidics offers advantages over traditional methods, such as higher encapsulation efficiencies and tighter control of LNP CQAs [20, 109]. Microfluidic mixing can be classified into two categories: passive and active. Passive microfluidic mixing is the most prominent method employed for LNP manufacture and usually emphasises natural fluid behaviour affected by microfluidic mixer design, while active mixing relies on external forces to enhance the mixing, such as ultrasonic or thermal signals [113]. Inclusions of advections within microfluidic mixers are often employed to induce a change to turbulent mixing enhancing nanoprecipitation, resulting in more desirable CQAs [20, 114]. A schematic of the different flows are shown in Figure 1-4. This thesis employs only passive mixing techniques, with

microfluidic mixing being a focus in Chapter 4. However, key features will be discussed here.

1.4.5.1 Reynolds Number and Péclet Number

In fluid dynamics, there are two numbers that help characterise the mixing mechanism: the Reynolds and Péclet numbers. The Reynolds number is dimensionless and indicates whether the flow is laminar or turbulent by comparing inertial to viscous forces. The equation is shown below [115]:

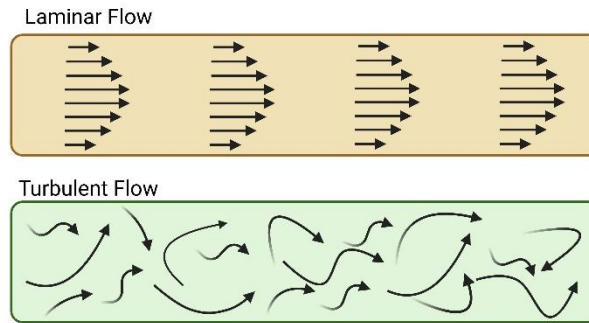


Figure 1-4 Simplified Schematic Representing the Differences in Turbulent and Laminar Flow Environments. Schematic created on Bio-Render.

$$Re = \frac{pDv}{\mu}$$

p = density of fluid

D = diameter of passage way

v = velocity of fluid

μ = viscosity of fluid

Passive microfluidic mixers usually exhibit laminar flow with Reynolds numbers between 1 and 100, and mixing times range from milliseconds to seconds. However, when the flow turns turbulent. Reynolds number increases to >2000, and the reaction time becomes <10 milliseconds [116].

The Péclets number is a dimensionless quantity that shows the ratio of the diffusion time scale [117]. When Péclets numbers are high, the mass transfer would be governed by advection, whereas low Péclets number mixing states rely on diffusion [118, 119]. The equation for Péclets number is shown below:

$$Pe = \frac{UL}{a}$$

U = characteristic speed

L = characteristic length

a = thermal diffusivity

1.4.5.2 Mixer Geometry

Microfluidic mixer geometry has also been highlighted as CPP in LNP manufacture [120, 121]. In addition, not only mixer design but mixer channel length and height can affect the formation of LNPs and liposomes [122]. Some of the key passive microfluidic mixing systems are described in Table 1-1.

Table 1-1 Passive Mixers Commonly Used in Nanoparticle Formulation. Information adapted from [120, 121]

Mixer Name	Type of Flow	Description	Advantages	Disadvantages
T-Mixer	Diffusion-Based Laminar Flow	The two fluids flow into the mixer perpendicular to each other, and nanoparticle production occurs at the organic:aqueous phase interface, with the output being the orthogonal channel	Relatively simple and low cost. Easy to use.	Typically require higher mixing speeds and wastage. Limited control over size unless system automation.
Impingement Jet Mixer	Turbulence induced Mixing	The Organic and Aqueous phases are directed towards each other at high velocities, which in turn increases chaotic mixing.	The mixers often perform better than diffusion-based mixers. Particularly effective at mixing high viscosity fluids.	The mixers carry the potential to cause shear stress if used at higher speeds. Increased susceptibility to channel clogging

				due to narrow channels.
Staggered Herringbone Mixer	Laminar Flow	Organic and Aqueous phases are injected simultaneously to flow through several herringbone structures to enhance diffusion-based mixing.	Mixing efficiency typically improved due to increased surface area. Offer more precise control over the size of nanoparticles.	Can be limited in production speeds. Typically, very expensive – company-dependent.
Toroidal Mixer	Chaotic Advection	Organic and Aqueous phases are injected simultaneously and flow around a series of obstacles designed to enhance mixing rates.	Can handle a wider range of flow rate ratios. Low wastage due to more efficient mixing.	High cost is associated – company dependent. Requires more precise fabrication techniques.
Cross-Flow Mixer	Diffusion-Based Laminar Flow	The organic phase is injected perpendicular to the continuous Aqueous phase and diffuses across the membrane to allow nanoparticle production.	Offers precise control over manufacturing parameters. Compatible with multiple lipid/payload materials.	More complex maintenance is typically required to prevent the membrane from clogging.
Pipette Mixing	Diffusion-Based Laminar Flow	The organic phase is pipetted into the aqueous phase, and nanoparticles form via diffusion.	Cheap and fast to manufacture. Can be done with relatively little training,	Extremely difficult to ensure consistent sizes, etc., due to a lack of precise control over manufacturing speeds.

1.5 Quality Control of Lipid Nanoparticles

The functionality of LNPs is closely linked to a range of critical quality attributes (CQAs), including sizes <200 nm, PDI <0.2, near-neutral surface charge, and high encapsulation efficiency. While regulatory guidance material does not always include specified values for LNP CQAs, smaller sizes are generally considered beneficial based off of previous research surrounding gene silencing with siRNA-LNPs [123]. Consequently, these CQAs require a consistent methodology to monitor them. In addition, researchers have started to identify a need for clear guidance on orthogonal techniques that could enhance determination [124]. By definition, orthogonal analysis involves the use of multiple different principles to

measure the same output, minimising bias [124, 125]. This section aims to investigate a range of LNP CQAs and how these are monitored during LNP formulation and manufacture.

1.5.1 Lipid Nanoparticle Size and Heterogeneity

Lipid nanoparticle size and heterogeneity are among the primary drivers of cellular trafficking and the *in vivo* fate of LNPs; therefore, accurate and reliable quantification is necessary for formulation success [126, 127]. Traditionally, LNP size is typically measured as a size distribution and represented as the hydrodynamic diameter. Both size and PDI are commonly measured using Dynamic Light Scattering (DLS), which quantifies nanoparticle size via Rayleigh or Mie scattering due to Brownian motion [128]. Both the EMA and FDA highlight DLS as an appropriate technique in their guidance materials, but the FDA also notes that complementary techniques may be needed to verify [129, 130]. Furthermore, while DLS is a reliable and established technique, it has several caveats, including the assumption that particles are spherical in shape, dilution of the sample is often required, and samples cannot scatter or fluoresce near 633 nm, as this is the wavelength of the equipped laser in a common DLS set-up [131]. Therefore, researchers have been developing orthogonal techniques to monitor LNP size and polydispersity. Nanoparticle Tracking Analysis (NTA) is one of the favourable techniques, as it monitors individual nanoparticle sizes in a continuous flow by measuring scattered light. This allows single-particle analysis and does not typically ‘shield’ larger particles, as DLS does [120]. In addition, flow field fractionation (FFF) is also gaining traction as a quality assessment tool. FFF is a separation technique based on LNP size and can be equipped with multi-angle light scattering (MALS) and UV detectors to provide information on LNP characteristics [132]. Research shown later in chapter 4 demonstrates that, while these orthogonal techniques do not always produce the exact same values, the trends are ultimately the same, highlighting the validity of these techniques.

1.5.2 Zeta Potential of Lipid Nanoparticles

Zeta potential is typically measured using Electrophoretic Light Scattering (ELS) and can be described as the electrical potential of a particle which is suspended in a liquid. Briefly, LNPs carry surface charges that attract opposite ions in solution, creating an adsorbed double layer. Zeta potential is a measure of the potential at the particle-fluid interface near the slipping plane [128]. The gold-standard measurement of zeta potential is ELS, with most researchers opting for it. Zeta potential is a key driver of LNP functionality and toxicity, with researchers reporting that neutral LNPs transfect more efficiently than positively or negatively charged LNP formulations [133]. However, other studies show that negative LNPs were more effective for T-cell delivery [134], highlighting that LNP characteristics require fine-tuning for optimal delivery.

1.5.3 Nucleic Acid Encapsulation and Mass Balance

Nucleic acid encapsulation and mass balance in LNPs is generally measured using a fluorescence-based assay such as Ribogreen™ [130]. The topic of RNA detection and quantification is explored more in Chapter 6. However, fluorescence assays have several drawbacks, including user-to-user variation, use of harmful materials and variation between RNA constructs [135, 136] and introduction of orthogonal techniques such as UV-Vis or HPLC detection can enhance RNA quantification, for example [137]. Additionally, cryo-TEM images are revealing that several LNP formulations are not truly spherical and are exhibiting bleb structures that have been shown to enhance efficacy [98]. Therefore, researchers have been establishing methods of identifying payload distribution. For example, Li et al. used laser cylindrical illumination confocal spectroscopy to monitor mRNA and lipid content, highlighting that empty LNPs and loaded LNPs could be easily distinguished [91].

1.5.4 Lipid Nanoparticle Storage and Stability

During the COVID-19 pandemic, limited timeframes led to LNPs being stored and transported under cold-chain conditions. However, this is not feasible for long-term use, and ideally, LNPs would be stable at room temperature. Therefore, it is vital that researchers have consistent, reliable methods to monitor the effects of storage on LNP stability and identify the ideal conditions. Loss of function during storage is due to degradation of the mRNA construct, the lipid components, or both [138]. mRNA degradation is likely to occur through loss of secondary structure, hydrolysis, or oxidation, all of which affect the base sequence and could result in no protein or altered protein production [138]. Lipid degradation can promote aggregation, resulting in payload leakage or lipid hydrolysis and the breakdown of LNPs [138]. Researchers have begun employing advanced techniques, such as molecular dynamics simulations, to elucidate LNP stability. For example, Hardianto et al., completed a study which looked at the effect of ethanol on LNP structure, with one of the key takeaways being the importance of purifying LNPs immediately [139]. In an attempt to improve formulation, researchers have also attempted to freeze-dry LNPs, which could then be stored at room temperature. This technique also has several drawbacks, as researchers have shown that lipid composition, formulation, buffer choice, cryoprotectant choice, and freeze-drying settings all need to be optimised to ensure that LNPs maintain good CQAs upon reconstitution [100, 140-142]. Furthermore, Schroder et al., conducted a study over LNP formulations across a temperature range and monitored structural changes using a mixture of NMR and molecular dynamics [143]. They indicated that the inner core and outer shells of LNPs freeze at different temperatures during the storage conditions [143]. Overall, current research highlights the difficulties associated with long-term LNP storage and stability, highlighting the need for optimised formulations that are able to withstand physical and chemical degradation.

1.6 Lipid Nanoparticle Mechanism of Action

While the success of LNPs is undeniable, formulations still require optimisation to enhance the amount of nucleic acid delivered to the cytosol and subsequently translated into the target protein. As it stands, only around 5% of nucleic acid is predicted to have successful endosomal release [68]. Increasing bioavailability could offer several advantages, including increased therapeutic effect and lower dosing. To begin optimising formulations to further enhance nucleic acid delivery, it is vital to characterise the mechanisms by which LNPs enter cells. Nucleic acid delivery occurs via endocytosis, as shown in the simplified schematic in Figure 1-5. Researchers have shown that Clathrin-mediated endocytosis (CME) is the primary pathway for LNP entry into cells. Gilleron et al. confirmed this by downregulating the heavy chains that form scaffolds during CME, resulting in a 50% reduction in LNP uptake [144]. Unspecific micropinocytosis has also been identified as an LNP entry route [144]. However, once inside the endosome, LNPs can either release mRNA for translation, undergo degradation or recycle back to the extracellular space [145]. Researchers are consistently working to tackle the endosomal escape bottleneck, and the key to enhancing it seems to lie in lipid modification. For example, Herrera et al. highlighted that LNPs containing β -sitosterol had increased endosomal perturbation compared to cholesterol-containing LNPs [60]. Through molecular dynamics studies, Singh et al. demonstrated that cone-shaped lipids were more successful in pore formation, as well as demonstrated that the nucleic acid distribution within the LNP can play a role in release kinetics [146]. This begins to highlight the need for increased endosomal escape to fully harness the capability of LNPs.

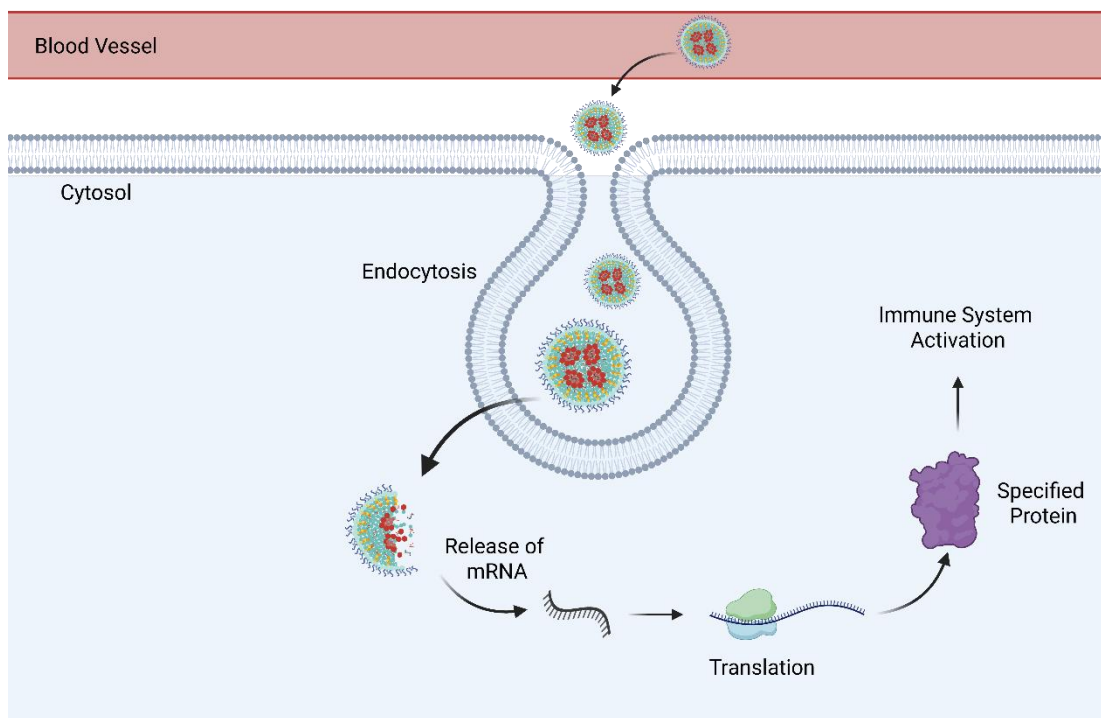


Figure 1-5 A Simplified Graphic Showing the Endosomal Escape of mRNA from Lipid Nanoparticles. Graphic was generated using Bio-Render.

1.7 Extra-Hepatic Targeting of LNPs

While LNPs have shown great clinical success, one of the major drawbacks associated include accumulation in the liver post-injection [147]. Following injection, serum proteins rapidly associate with the cell surface, mainly ApoE [147, 148]. The ApoE protein then endogenously targets the LDL-R receptor present on liver cells, therefore increasing uptake [148]. Furthermore, Sebastiani et al. demonstrated that the binding of ApoE could cause lipid rearrangement and aid endosomal escape [149]. However, it should be noted that current LNP formulation research is based on research leading to Onpatro, which required effective delivery to the liver; this means that ways to tackle extrahepatic targeting are now one of the main bottlenecks in alternative LNP use [148].

1.7.1 Active Targeting of Lipid Nanoparticles

Active targeting of LNPs is defined as the modification of the LNP to include functionalised ligands that can bind targets on specific tissues or cells [150]. Furthermore, active targeting can be done through post-insertion during manufacture, and targeted ligands can vary largely from sugars to antibodies [150]. Active targeting will be explored more in Chapter 5, and the use of target ligands can aid in increasing immune cell uptake or trafficking to specific organs [150]. For example, Anthiya et al. formulated T-Lyp-1 functionalised LNPs that encapsulated siRNA anti-KRAS and demonstrated that, in combination with first-line chemotherapies, a significant reduction in pancreatic tumour growth was detected [151]. However, caution is warranted when using surface modification methods due to the risks associated with physicochemical changes. For example, Swart et al. demonstrated that post-insertion of ligand-lipid conjugates had better preservation of characteristics compared to surface modification [152].

1.7.2 Passive Targeting of Lipid Nanoparticles

Passive targeting of LNPs is achieved by altering components that change their physicochemical characteristics [150]. Many passive-targeting forms of liposomes have been developed to target tumour environments. Most prominent would be Doxil – which exhibits a stealth liposome formulation to enhance passive targeting via enhanced permeability and retention effects [153]. Furthermore, Silva et al., demonstrated that liposomes loaded with hypoxia-targeted therapies exhibited superior *in vitro* performance in prostate cancer models [154, 155]. The most advanced form of passive targeting is through the use of Selective Organ-Targeting (SORT) LNPs, in which the lipid components are altered to result in changes to the LNP charge, which could drive organ specificity [156]. Wang et al. showed that LNPs could be targeted to the lungs, spleen and liver through the use of cationic, anionic or ionisable lipids, respectively [156].

Furthermore, Cheng et al. then highlighted that this effect was not formulation-dependent [157]. This opens up an easy and cheap method of LNP targeting that could be exploited to enhance extrahepatic delivery. Furthermore, researchers have also begun targeting LNPs using active incorporation of endogenous vitamins that can then act in a passive targeting manner. For example, Isaac et al., completed a study where they including an endogenous vitamin (either A, B2, D3, E or K1) in a LNP formulation to determine the organ targeting effects, highlighting that inclusion of vitamin D3 can aid in targeting LNPs from the liver to the pancreas [158]. Additionally, Jester et al., highlighting the potential of LNPs with a modified PEG-folate for targeted treatment in cancer cells [159]. This targeting method is beneficial to researchers as it utilises existing biological receptor-mediated uptake offering increased efficacy. However, it is also important to recognise that they could lack specificity which can limit their use.

1.8 Clinical Applications of LNPs

LNPs have enabled the delivery of challenging therapeutics through their enhanced payload protection and adaptable formulations. As a result, LNPs have become a key player in pharmaceutical research. This section will explore differences among currently approved LNP formulations and future perspectives.

1.8.1 Current Approved LNP Formulations

There are currently four approved nucleic acid-LNP formulations on the market, with their key similarities and differences shown in Table 1-2.

Table 1-2 Details of the Current Approved LNP Formulations. Chol = cholesterol, I.V. = intravenous, I.M. = intramuscular. Information adapted from [102, 160].

Name/Company	Onpattro - Alnylam	Comirnaty – Pfizer-BioNTech	mRNA-1273 - Moderna	mRESVIA - Moderna
Nucleic Acid	siRNA	mRNA	mRNA	mRNA
Composition	10:38.5:50:1.5% (DSPC:Chol:MC3:DMG-PEG2000)	9.4:42.7:46.3:1.6% (DSPC:Chol:ALC-0315:ALC-0915)	10:38.5:50:1.5% (DSPC:Chol:SM-102:DMG-PEG2000)	Unknown
N/P Ratio	3	6	6	Unknown
Dosage	0.3 mg/kg I.V	30 µg I.M.	100 µg I.M.	50 µg I.M.
Target Protein	Transthyretin protein	Spike protein (S) of SARS-CoV-2	Spike protein (S) of SARS-CoV-2	RSV-A glycoprotein F

The main difference between the approved formulations lies in their uses. Onpattro was designed to encapsulate siRNA that can downregulate the transthyretin protein in patients with hereditary transthyretin amyloidosis (hATTR), while Comirnaty and mRNA-1273 both encode a targeted S-Spike protein for COVID-19, and the most recent approval, mRESVIA, encodes an RSV-A glycoprotein F for the treatment of RSV [88]. Table 1-2 highlights that all four vaccines require different dosages, which is not ideal, as it reduces the flexibility of LNP formulations to encapsulate different mRNA constructs. The differences in doses could be due to formulation efficacy. Onpattro employs the ionisable lipid DLin-MC3-DMA, which consists of two 18-carbon linoleyl chains linked by an ester to a dimethylamino head group [68]. Whereas mRNA-1273 and mRESVIA contain SM-102, Comirnaty uses ALC-0315. Both of these ionisable lipids have 14-carbon dialkyl chains bound by ester linkage locations to tertiary amine head groups; however, ALC-0315 has a double-branched structure compared to SM-102, which exhibited only a single-branched structure [88, 161] – these are further displayed in a schematic in Chapter 3. Comirnaty also contains an ALC-0315 PEGylated lipid, differing from other approved formulations. All LNPs exhibited pKa values between 6 and 6.7, and the inclusion of ester linkages

allows hydrolysis, increasing clearance and enhancing biodegradability [68, 88, 161]. As a concluding point, the majority of formulations follow the standard molar ratio of 10:38.5:50:1.5%, with Comirnaty opting for altered levels; however, the change is minor, and the effect of the molar ratio is explored further in Chapter 3.

1.8.2 The Future Landscape of Lipid Nanoparticles

Following this clinical success, LNPs are now among the most promising pharmaceutical drug products. The ability of mRNA to be tailored to virtually any target protein gives mRNA-LNP vaccines a versatility that is hard to replicate [162]. A scan of the ClinicalTrials.gov website was used to generate a schematic shown in Figure 1-6. Around 38% of clinical trials have focused on COVID-19, with the landscape shifting to cancer (17%) and infectious disease (12%) (Figure 1-6). The 33% of other treatments cover a range of diseases, including diabetes, bone regeneration and cystic fibrosis [160]. Together, the currently approved therapies and ongoing clinical trials highlight the growing potential of LNPs and demonstrate the platform's versatility.

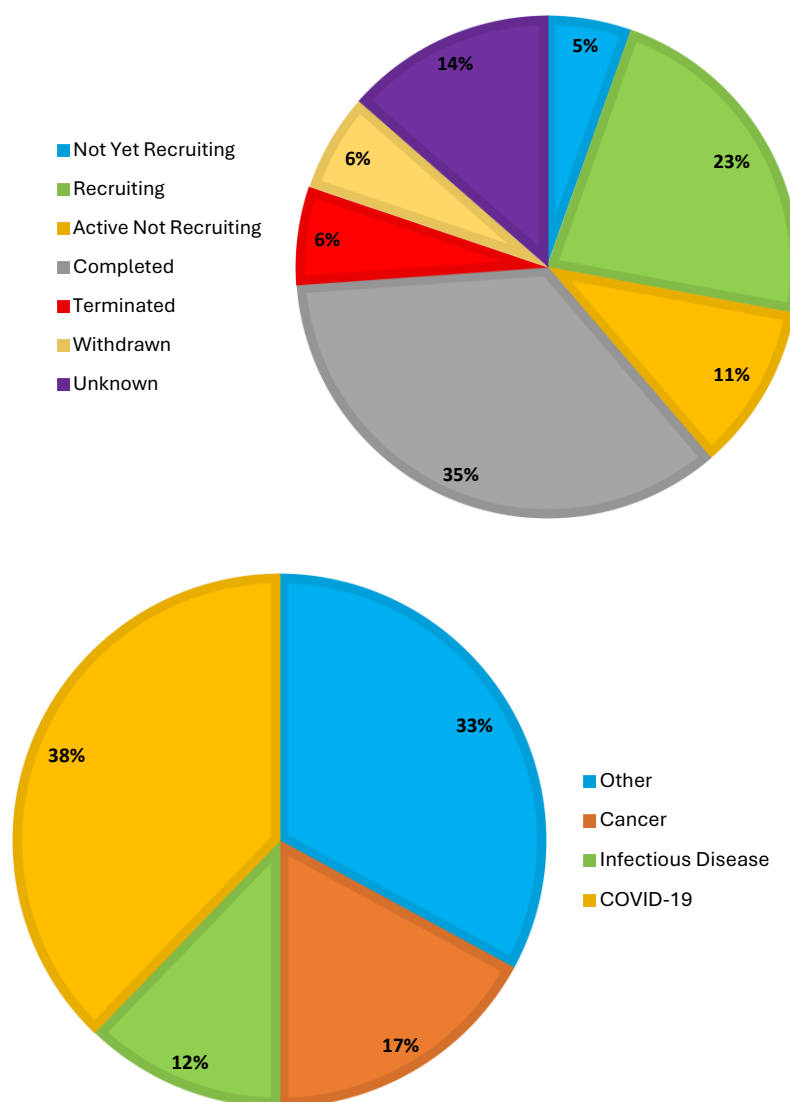


Figure 1-6 The Clinical Landscape of Lipid Nanoparticles. A search of the intervention term 'lipid nanoparticle' gathered 111 studies which were categorised based on their status (a). The clinical trials that were either, not yet recruiting, recruiting, active but not recruiting and completed were further characterised based on their treatment (b). This information is up to date as of Thursday 2nd April 2026.

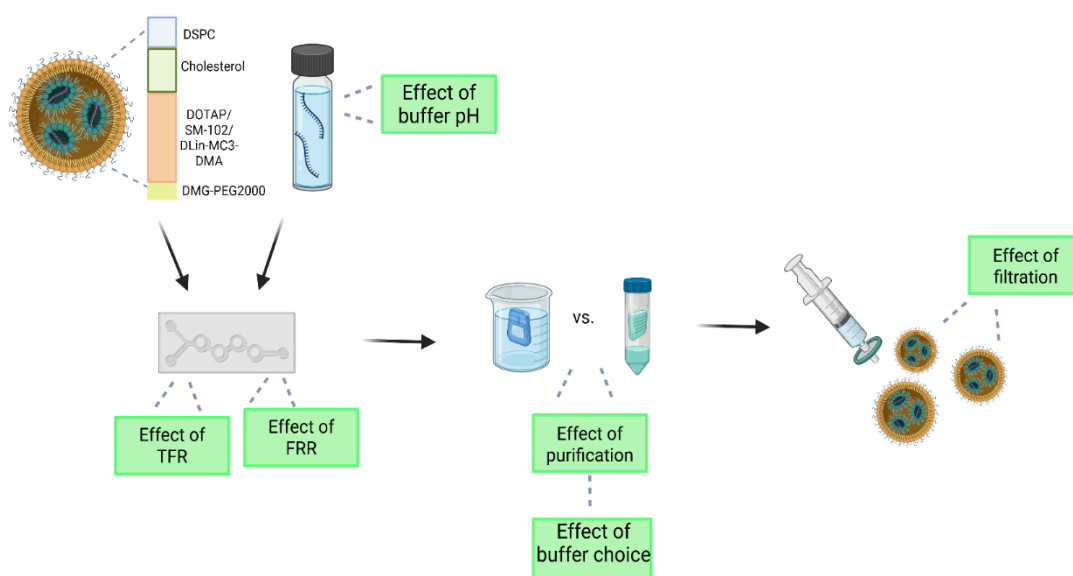
1.9 Aims and Objectives

The overall aim of the work in this thesis was to develop enhanced formulation screening and manufacturing techniques that could increase the accessibility of mRNA-LNP vaccines. LNP formulation screening was enhanced by increasing knowledge of effective screening methodologies, while microfluidic manufacturing was enhanced by selecting optimal critical process parameters and low-cost alternatives. Additionally, orthogonal techniques were employed to enhance the characterisation of LNPs, thereby increasing the reliability of conventional methods. Finally, optimisation was considered when targeted studies were undertaken to demonstrate the selectivity of functionalised and standard LNP formulations.

To achieve these aims, the objectives were;

1. To investigate the effect of critical process parameters (CPPs) during microfluidic manufacture (including wastage, TFR, FRR and lipid concentration) to determine their effect on particle size, polydispersity, charge and encapsulation efficiencies.
2. To compare OFAT as well as manual and automated DoE methods in formulation screening to determine the fastest and most reliable screening tool.
3. To evaluate the efficacy of low-cost microfluidic mixers as well as establish them as reliable screening tools that ensure precision, accuracy and repeatability.
4. To develop and evaluate the *in vitro* and *in vivo* performance of a functionalised LNP formulation aimed at targeting macrophage cell populations.
5. To investigate alternative analytical tools for monitoring the encapsulation and biological efficacy of mRNA within LNP formulations.

Chapter 2: Process Perfection: How Well-Defined Manufacturing Parameters Can Play a Role in LNP Success



Type of Publication	☐ Published article ☐ Accepted for publication ☐ Submitted for publication ☐ Drafted for publication/application notes ☒ Not applicable
Citation Details (only applicable if published)	N/A
Chapter Inclusion Details	☐ This chapter is a word-for-word reproduction of the published article ☐ This chapter contains minor revision of the original publication ☐ This majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	N/A

2.1 Introduction

The manufacture of LNPs comprises multiple stages, including (but not limited to) phase preparation, microfluidic mixing, purification, and fill-and-finish under aseptic conditions. Each area of manufacture comes with its own set of Critical Process Parameters (CPPs), which can determine the LNP physicochemical attributes [77, 105, 106, 163-165]. The predominant CPPs for each stage are shown in Figure 2-1.

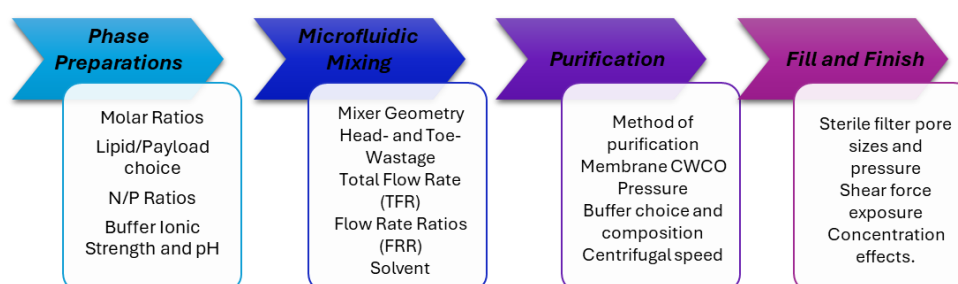


Figure 2-1 The Critical Process Parameters of LNP Manufacturing. This flow diagram represents a selection of key CPPs at varying steps of LNP manufacture. Diagram was created using Microsoft PowerPoint v2601 Build 16.0.19628/20214).

Due to the nature of their phase components and manufacturing conditions, LNPs are highly sensitive to small changes, leading to alterations in their physicochemical attributes. This highlights the need not only to optimise techniques but also for a true understanding of the overall process. Due to the intricacies of each step, it is increasingly difficult to attribute a change to a single factor. Instead, it is beneficial to consider the variables as interacting groups.

Phase preparation is often the key driver of LNP architecture. The choice of lipids and payload will determine the LNP composition, with different lipids offering precise size control [166] and altered efficacy [77], while different payloads will not only offer different therapeutic effects but also require different lipid compositions [83]. In addition, the choice of aqueous buffer can severely affect LNP formation, with ionic strength being a key driver of electrostatic interactions between the nucleic acid payloads and ionizable lipids, thereby affecting

encapsulation [167]. Additionally, when determining lipid choice, the N/P ratio must be considered as the reaction between the nitrogen of the tertiary amine and the phosphate of the nucleic acid backbone is the key process by which payloads are encapsulated, and insufficient ratios could lead to insufficient binding potential and a lack of complexation [168].

Microfluidic mixing is the key process which controls how the lipids assemble during manufacture. Microfluidics offers a laminar flow environment in which the lipid-rich ethanol phase and payload-rich aqueous phase will assemble into LNPs, where the CQAs are governed by the mixing speeds and ratios [112]. Higher mixing speeds offer faster solvent dilution rates, which can often lead to smaller, more homogenous LNP populations [169]. Similarly, the mixer geometry is also a key driver of the efficacy of the LNP assembly. With both low-cost laminar flow mixers [165] and mixers which harness grooves or advections to generate chaotic flow [20] being shown to successfully produce LNPs within a range of standard CQAs.

The two prior stages aid in governing LNP architecture and formulation; however, the final purification and fill-finish steps are key drivers of LNP stability and batch clinical acceptance. Purification of LNPs can be carried out via four main methods: in-line dilution, dialysis, spin column chromatography and tangential flow filtration (TFF), each of which results in differences in pH change rates and addition of shear or centrifugal forces, which could affect the resulting LNP. Furthermore, for clinical acceptance, it is a necessity that LNPs are sterile filtered generally through a 0.22 μ M filter, which could result in the larger LNPs being retained and cause issues for population CQAs [170].

Overall, it is evident that each CPP has the potential to disrupt the fine-tuned LNP formulation, and care must be taken when selecting CPPs for optimised runs. Therefore, in this chapter, a selection of key CPPs were investigated with the aim of understanding their effect on the critical quality attributes of LNPs, with the

learning objectives of evaluating how CPPs can affect hydrodynamic mixing in microfluidic devices and enhancing knowledge of how formulation and composition can affect the CQAs of LNPs.

2.2 Materials and Methods

2.2.1 Experimental Materials

The cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), the phospholipid 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and the PEGylated lipid 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were acquired from Avanti Polar Lipids (Alabaster, AL, USA). While the Ionizable lipids Heptadecan-9-yl 8-[2-hydroxyethyl-(6-oxo-6-undecyloxyhexyl)amino]octanoate (SM-102) and (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA) were acquired from Broadpharm (San Diego, CA, USA). Cholesterol, Triton X-100, sodium citrate dihydrate, citric acid, Diethyl Pyrocarbonate (DEPC) and Polyadenylic Acid (Poly A) were purchased from Merck Life Science UK Ltd (Gillingham, UK). 20X TE buffer, Quanti-iT™ Ribogreen™ Assay Kit, Tris base and Sucrose were all purchased from Thermo Fisher Scientific (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). EZ Cap™ Firefly Luciferase mRNA (5-moUTP) and EZ Cap™ Cy5 Firefly Luciferase mRNA (5-moUTP) were purchased from APEX BIO (Houston, TX, USA). CPI (Centre for Process Innovation, Sedgefield, UK) supplied Green Lantern mRNA and Fusion mRNA. The ONE-Glo™ Luciferase Assay System and VivoGlo™ Luciferin were acquired from Promega Ltd. (Chilworth, UK). Ethanol was supplied by Supelco (Bellefonte, PA, USA), and Ultrapure water was supplied through an in-house system. Finally, HEK-293 cells were purchased from American Tissue Culture Collection (ATCC) (CRL-1573).

2.2.2 Microfluidic Mixer Set Up

Throughout this chapter, lipid nanoparticles were manufactured on a range of microfluidic mixers with a total flow rate (TFR) of 15 mL/min and a flow rate ratio

of 3:1 (aqueous:lipid) unless otherwise stated. LNPs manufactured on the NanoAssemblr® (Cytiva, Vancouver, Canada) with a staggered herringbone mixer (SHM) had a head and toe cut off of 300 μ L and 50 μ L. LNPs manufactured on the Nova Benchtop (Helix Biotech) were prepared on the size 1 mixer with start and end cut-offs of 450 μ L and 50 μ L, respectively. The T-junction, Confined Impingement Jet mixer and their corresponding automated pump control system were set up as described in [165]. The AXF-Mini mixer was also controlled using the same automation system with a head cut off of 500 μ L, and no tail cut off. Any alterations to wastage, TFR or FRR will be detailed in figure legends.

2.2.3 Preparation of Aqueous Phases

Aqueous phases consisted of pH 4 or pH 5 citrate buffer (50 mM) and either Poly A, Firefly luciferase mRNA (Fluc), Green Lantern mRNA (GrL) or Cy5-Labelled Firefly luciferase mRNA (Cy5-Fluc). Final payload concentrations were 0.055 mg/mL unless otherwise stated, and phases were prepared to account for a nitrogen:phosphate (N/P) ratio of 6.

2.2.4 Preparation of Lipid Phases

Lipid phases consisted of DSPC, cholesterol and DMG-PEG2000 alongside a range of cationic/ionizable lipids (SM-102, DOTAP, DLin-MC3-DMA) diluted in HPLC-grade absolute ethanol (>99.5%). Molar ratios consisted of 10% DSPC, 38.5% cholesterol, 50% ionizable/cationic lipid, and 1.5% DMG-PEG2000; any deviations from the standard molar ratio are detailed in the figure legends along with the final lipid concentration which ranged between 0.28-1.25 mg/mL, which was calculated to maintain a N/P ratio of 6.

2.2.5 Lipid Nanoparticle Purification

Post-manufacturing LNPs were purified using either dialysis or spin column chromatography. Dialysis purification consisted of LNPs being dialysed against 10 mM pH 7.5 Tris buffer or PBS for 1-4 h using dialysis tubing with a molecular weight cut-off of 14 kDa. Spin column purification was carried out by diluting

LNPs 1:40 with 10 mM pH 7.5 Tris buffer, and centrifugal filtration was carried out using Amicon Ultra-15 columns at 2000× g at 4 °C until the desired volume was reached.

2.2.6 Lipid Nanoparticle Characterisation

LNP Z-average diameter (nm), polydispersity index and zeta potential (mv) were characterised using either the Zetasizer Nano or Zetasizer Ultra (Malvern Panalytical, Ltd., Worcestershire, UK). For analysis, LNPs were diluted to a lipid concentration of 0.1 mg/mL. Size and polydispersity index measurements were diluted in citrate buffer (viscosity 1.28 cP, refractive index 1.47), PBS (viscosity 1.02 cP, refractive index 1.335) or Tris buffer (viscosity 1.0037 cP, refractive index 1.47) while zeta potential measurements were diluted in water (viscosity 0.8872 cP, refractive index 1.33).

Payload encapsulation and mass balance were monitored using Quanti-iT™ Ribogreen™ Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Low (0-50 or 0-200 ng/mL) or high (0-1000 ng/mL) curves were generated using the appropriate encapsulated material and used to quantify encapsulated payload from intact LNPs (-Triton) and burst LNPs (+Triton). For analysis, LNPs were diluted to 3 µg/mL and mixed with either 50 µL 1X TE buffer or 2% 1X Triton-TE buffer and incubated at 37 °C for 15 minutes. Post-incubation 100 µL of a 200X (+Triton wells) or 500X (-Triton wells) Ribogreen™ reagent mixture was added to account for a 1:1 dilution. Fluorescence signals were measured using the Glomax Plate Reader (Promega, Chilworth, UK) with excitation at 475 nm and emission at 500-550 nm or on the POLARstar OMEGA Microplate reader (BMG LABTECH, Ortenberg, Germany) at an excitation of 475 nm and emission of 550 nm.

2.2.7 Syringe Filtration

Samples that were subjected to filtration were passed through a 0.22 µm PES filter. The filter was either maintained in initial condition (dry) or had 10 mL of Tris buffer 10mM pH 7.5, syringed through (Wet) prior to any LNP samples.

2.2.8 Cell Viability

HEK-293 cells were seeded in a clear 96-well plate at a concentration of 1.5×10^4 cells/well and cultured for 48 hours in a 37 °C 5% CO₂ environment. LNP treatment was carried out in triplicate wells at four mRNA concentration ranges: 2 – 1 – 0.5 and 0.25 µg/mL. Cells were left for a further 48 hours at 37 °C, 5% CO₂ and on the day of analysis, 10 µL of resazurin was added to all wells, and the plate was incubated for a further 4 hours before being measured on the GloMax® Plate Reader (Promega, Chilworth, UK) at excitation 520 nm and emission 580-640 nm.

2.2.9 *In vitro* Protein Expression

For Fluc mRNA protein expression, HEK-293 cells were seeded in a white, clear-bottom 96-well plate at a concentration of 1.5×10^4 cells/well and maintained at 37 °C, 5% CO₂ for 48 hours. Cells were treated with LNPs in triplicate at concentrations ranging from 2 to 0.25 µg/mL and cultured for an additional 48 hours. On the day of analysis, 100 µL of One Glo was added to all wells, and results were measured on the Glomax Plate Reader (Promega, Chilworth, UK) using the luminescence filter. For cells treated with GrL-LNPs to express Green Fluorescent Protein (GFP) images were captured on the EVOS M5000 microscope using the trans and GFP bulbs.

2.2.10 *In vivo* Protein Expression Studies

To evaluate the *in vivo* biodistribution and expression efficiency of the mRNA-LNPs with various fluorescent detection labels, 9-11-week-old male BALB/c mice were injected bilaterally with 50 µL/leg of mRNA-LNPs intramuscularly equating to 7 µg of firefly luciferase mRNA per mouse (3.5 µg/mL per leg). Fluorescence signals from the DiR/Cy5 incorporated into the formulation were detected by the

IVIS Spectrum (PerkinElmer) instrument measurement under the DiR fluorescence filter excitation 710 nm and emission 780 nm, and Cy5 Fluorescence filter excitation 649 nm and emission 666 nm. The mice were then subcutaneously injected with VivoGlo D-Luciferin substrate (30 mg/mL), corresponding to 5 μ L/g of D-luciferin, and bioluminescence signals were detected using the IVIS Spectrum (PerkinElmer) instrument. All imaging was conducted 3 and 6 hours post LNP injection. At the 6h time point, after imaging, the mice were culled, and the liver and spleen were removed for ex vivo determination of bioluminescence and fluorescence signals. The fluorescence and bioluminescence signal analysis was done using the Living Image 4.0 software.

To evaluate the effect of TFR on *in vivo* biodistribution, 14-weeks old female BALB/c mice were injected bilaterally with 50 μ L/leg of mRNA-LNPs intramuscularly equating to 2 μ g of firefly luciferase mRNA per mouse (1 μ g/mL per leg). Mice were imaged at 0.5, 6, and 24 hours post-injection, immediately following subcutaneous injection of Vivo Glo D-luciferin substrate (30 mg/mL), equating to 5 μ L/g, and bioluminescence signals were detected using the IVIS Spectrum (PerkinElmer) instrument. Image analysis and signal quantification was carried out using Living Image Software v4.0.

2.2.11 Capillary Gel Electrophoresis

To extract mRNA from LNP samples, samples were diluted to achieve 100 μ L at 10 μ g/mL in tris buffer. 750 μ L of ethanol and 25 μ L of 3 M sodium acetate buffer pH 8 was added and samples were centrifuged at 14,000 rpm for 20 minutes. Supernatant was carefully removed and ethanol precipitation was repeated once more. Final mRNA pellet was resuspended in 35 μ L of RNase free water. 11.3 μ L of formaldehyde loading dye was added (1:3 v/v). The positive control was generated by diluting eGFP to achieve 35 μ L at 10 μ g/mL with addition of 11.3 μ L formaldehyde loading dye while 3 μ L of the RNA Millenium Marker and 2 μ L of ultrapure water were mixed with 15 μ L formaldehyde loading dye. All mixtures

were denatured at 65°C for 10 minutes. A 1% agarose gel was made by combining 0.5 grams of agarose powder with 50 mL 1X MOPs buffer and heated until uniform suspension achieved and once solution had cooled to 55°C, 5 µL of SYBR green dye was added. Gel was allowed to set for 30 minutes prior to use. 7 µL of each sample was added to the wells and the gel was run at 90 V for approximately one hour until the samples had moved through ~75% of the gel. Images were acquired on the Gel Doc EZ Imaging System (Bio-Rad, CA, USA).

2.2.12 Statistical Analysis

All statistical analysis was carried out using Minitab v19 or v22. Data was checked for normality using an Anderson-Darling normality test. All parametric data were analysed using a one-way ANOVA with Tukey's post-hoc, and non-parametric data were analysed using the Kruskal-Wallis test, followed by a T-test if significance was identified.

2.3 Results

2.3.1 The Effect of Formulation Parameters

Fine-tuning the structure and function of LNPs typically begins with formulation modification. By adjusting the lipid components such as the ionizable lipids, phospholipids and PEGylated lipids, it is possible to precisely tailor CQAs such as decreased size and increased encapsulation, while modifying the encapsulated payload will modulate the therapeutic effect. Any modifications are made with the aim of enhancing efficacy, safety, and ease of manufacture, and reducing off-target effects; however, to truly optimise LNP formulations, it is essential to understand how formulation parameters affect the resulting LNPs. Therefore, this results section will analyse how key parameters, such as lipid choice, concentration, and buffer choice, affect formulation CQAs.

2.3.1.1 Choosing Ionizable-Cationic Lipids

Initial research focused on how altering the ionizable-cationic lipid can affect the LNP CQAs, pre- and post-purification. To analyse this, two commercially

available ionizable lipids (SM-102 and DLin-MC3-DMA) and one cationic lipid (DOTAP) were used alongside DSPC, cholesterol and DMG-PEG2000, at a molar ratio of 50:10:38.5:1.5% respectively, to manufacture LNPs, which were then analysed for particle size, homogeneity, charge and payload encapsulation/mass balance. Figure 2-2 highlights any key differences between the CQAs of the formulations, showing that DOTAP-LNPs result in significantly ($p<0.05$) smaller sizes than both SM-102- and DLin-MC3-DMA-LNPs post-purification (Figure 2-2a). In addition, DOTAP-LNPs demonstrate significantly increased heterogeneity compared to SM-102-LNPs (Figure 2-2a) with no significant differences in charge witnessed (Figure 2-2b). DLin-MC3-DMA-LNPs exhibited a significantly ($p<0.05$) decreased poly A mass balance compared to DOTAP- or SM-102-LNPs. These results highlight how small changes to formulation can begin to govern the characteristics of LNPs.

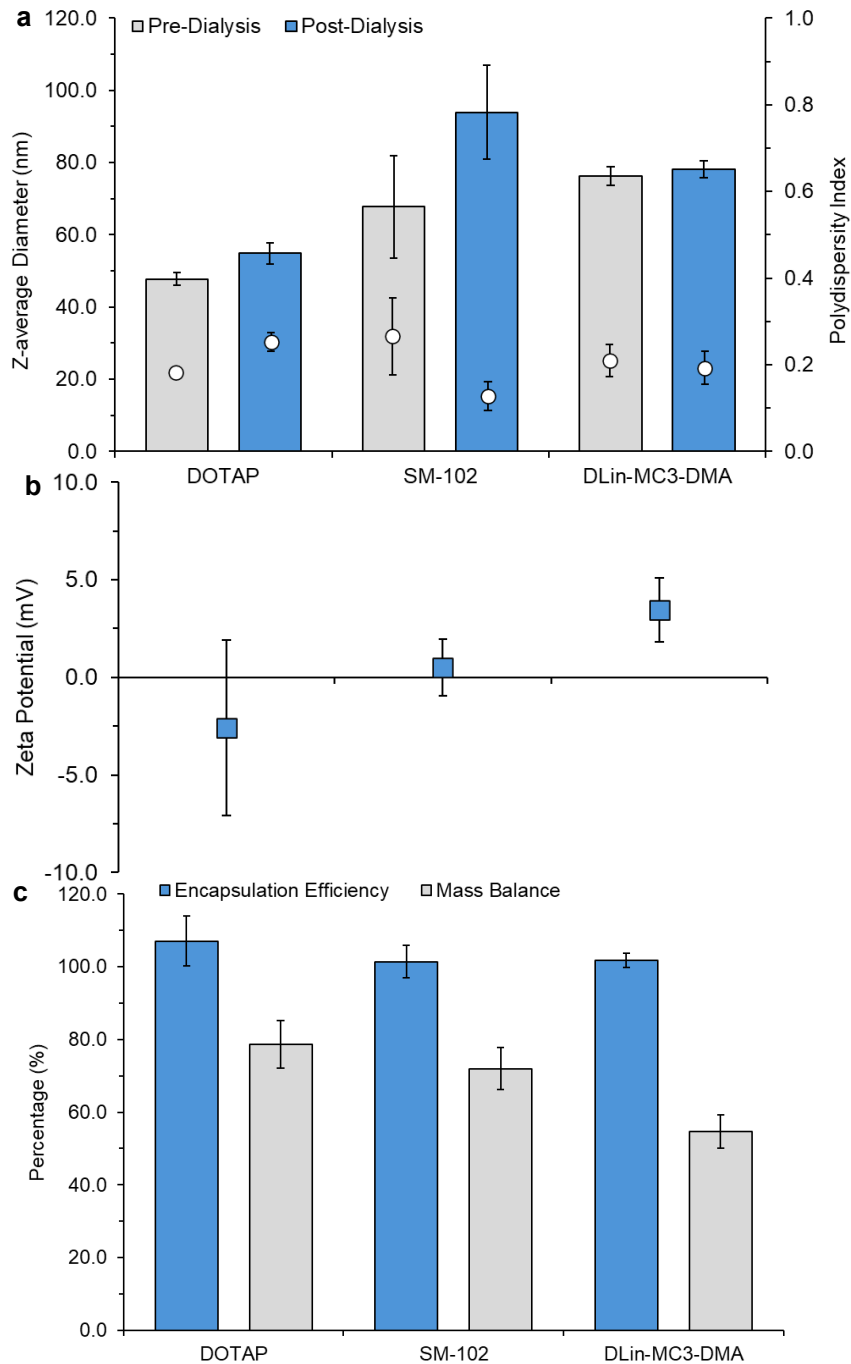


Figure 2-2 The Effect of Ionizable-Cationic Lipid on LNP CQAs. LNPs consisted of DSPC:Cholesterol:DMG-PEG₂₀₀₀:DOTAP or SM-102 or DLin-MC3-DMA encapsulating poly A to a final concentration of 0.055 mg/mL and purified by dialysis. (a) represents that LNP size and homogeneity pre- and post-dialysis while (b) represents the surface charge post-purification. Payload encapsulation and mass balance is represented in (c). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

2.3.1.2 Determining Lipid Concentration

In addition to lipid choice, the concentration of the lipid mix can alter CQAs, leading to discrepancies in formulation efficacy. Here, initial lipid concentrations ranging from 2.2 to 6.8 mg/mL were investigated as a factor across varied microfluidic mixers, and LNPs were subjected to standard characterisation testing.

Figure 2-3 demonstrates the effect of lipid concentration using three varied microfluidic mixers: a T-junction mixer, a Confined Impingement Jet mixer (IJM) and the AXF-Mini mixer, showing that for all mixers, LNPs sizes ranged between 95-122 nm with PDI < 0.02 and near neutral surface charges between -2.1 and 1.1 mV. Additionally, all LNPs had high encapsulation (>95%) and between 50 - 61% poly A mass balance. These results suggest that slight variations in lipid concentrations have no effect on the CQAs; however, different patterns emerge across mixers. For example, both the IJM and AXF show decreases in size as lipid concentration is decreased, whereas the opposite is true for the T-junction mixer. Indicating that LNP formulations have the potential to behave differently depending on their manufacturing parameters.

Building on this, Figure 2-4 illustrates the effect of lipid concentration across a range of TFRs for formulations manufactured using the Impinging Jet mixer – Nova Benchtop (Helix Biotech, TN, USA). Figure 2-4a shows that, as lipid concentration increases, the pre-purification particle size decreases slightly, although the trend is not statistically significant across all TFRs. Similarly, the pre-purification PDI at the higher lipid concentration of 6.8 mg/mL and faster TFR of 15 mL/min was increased compared to the others at a value of 0.224. Additionally, at lower lipid concentrations, higher TFRs resulted in increased zeta potential; however, all LNPs remained near neutral, ranging from 0.2 to 6.7 mV (Figure 2-4b). Finally, Figure 2-4c showed that the varied parameters had no effect on poly A encapsulation or mass balance.

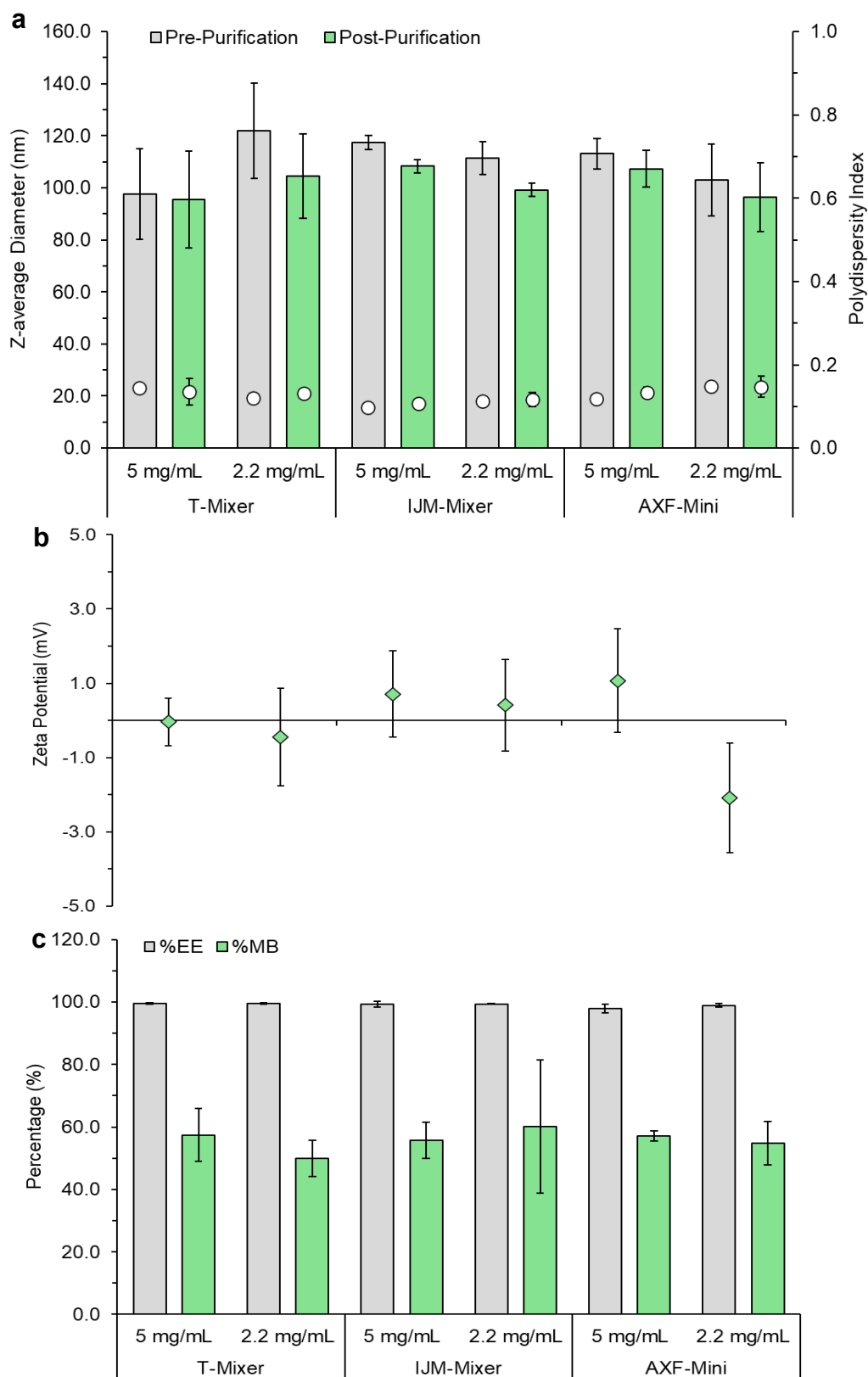


Figure 2-3 The Effect of Lipid Concentration on LNP CQAs. LNPs consisted of DSPC:Cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating poly A to either a final concentration of 0.055 mg/mL or 0.025 mg/mL to equate for an initial lipid concentration of 5 mg/mL or 2.2 mg/mL and purified by dialysis. (a) represents the size (nm) and homogeneity pre- and post-dialysis while (b) represents the zeta potential (mV) and (c) represents poly A encapsulation efficiency (%EE) and recover (%MB). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

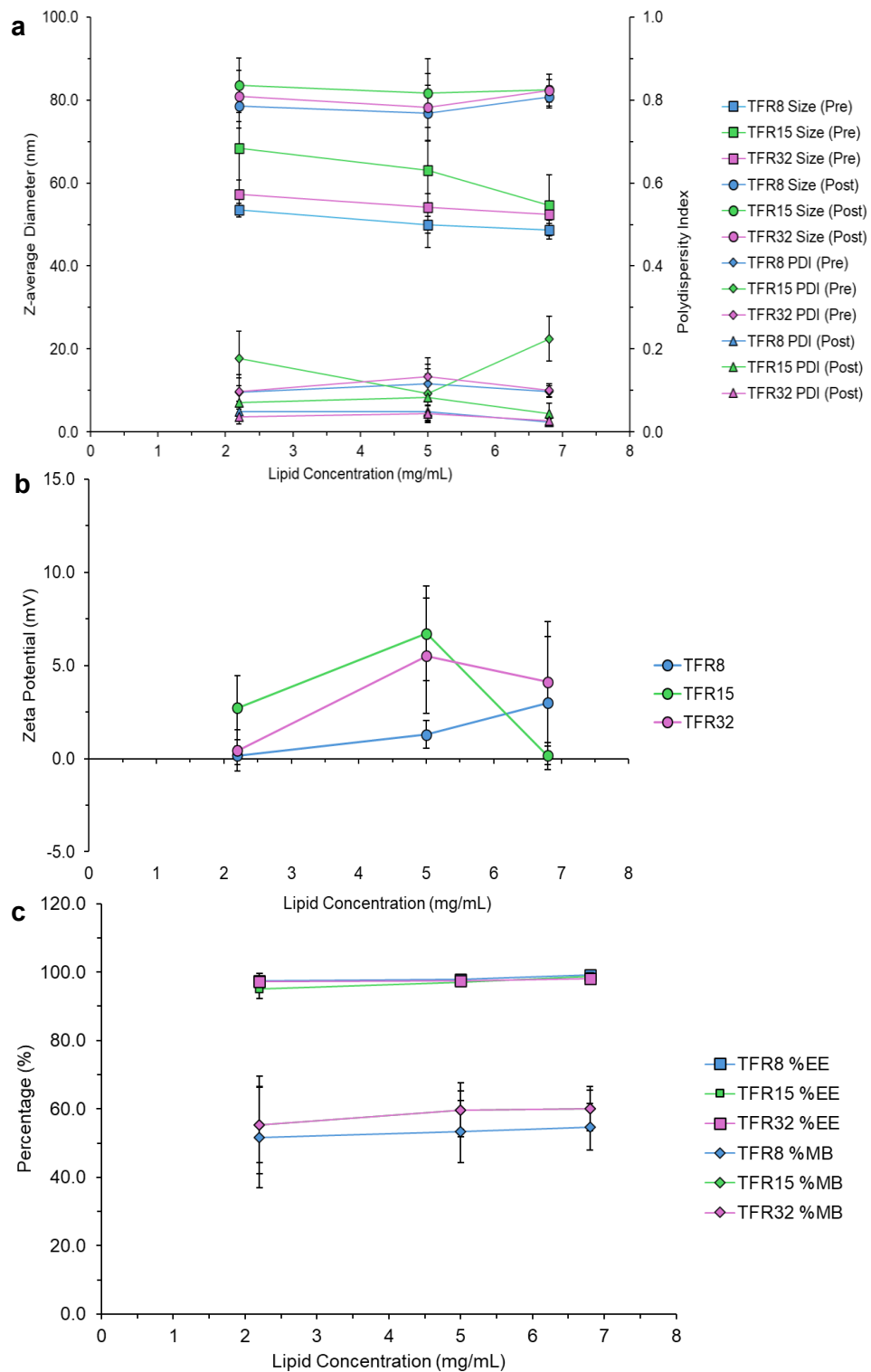


Figure 2-4 The Effect of Lipid Concentration at Varied TFRs. LNPs consisted of DSPC:Cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating poly A to a final concentration of 0.025, 0.055 or 0.075 mg/mL and purified by dialysis. (a) consists of the trend of size (nm) and polydispersity over a range of TFRs pre- and post-purification while (b) indicates the zeta potential (mV) and (c) reports the poly A encapsulation efficiency (%EE) and mass balance (%MB). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

2.3.1.3 Altering Aqueous Buffer pH

The next manufacturing parameter investigated was the effect of aqueous phase buffer pH. As mentioned in the introduction, acidic buffers are essential for ionizable lipids to function correctly, as these lipids require adequate protonation to encapsulate nucleic acids. However, mRNA is also unstable in acidic environments over extended periods due to an increased risk of hydrolysis. Highlighting that there is a fine balance between protonation and stability when it comes to deciding on aqueous buffer pH. To analyse this, LNPs were manufactured with aqueous phases consisting of either a poly A pH 4 citrate buffer or a pH 5 citrate buffer. Figure 2-5 shows the effect of aqueous phase buffer on three formulations with varied molar ratios.

The results demonstrate that changing from pH 4 to pH 5 of the aqueous buffer has no significant effects on size, charge, or poly A mass balance and encapsulation pre- and post-purification. All LNPs had sizes ranging from 77 to 106 nm, zeta potentials between 8 and 15.1 mV, poly A encapsulation >93%, and mass balance between 42 and 70% (Figure 2-5). The only significant increase was seen for the post-purification PDI of formulation 1 (48.5:0:50:1.5%), 0.295 in pH 4 buffer compared to 0.397 in pH 5 (Figure 2-5a). However, formulation 1 consistently exhibits an unacceptable PDI range, possibly due to a lack of cholesterol, which weakens the lipid layer. Notwithstanding, this significant difference is likely not clinically relevant. The remaining formulations experienced no significant changes to LNP homogeneity.

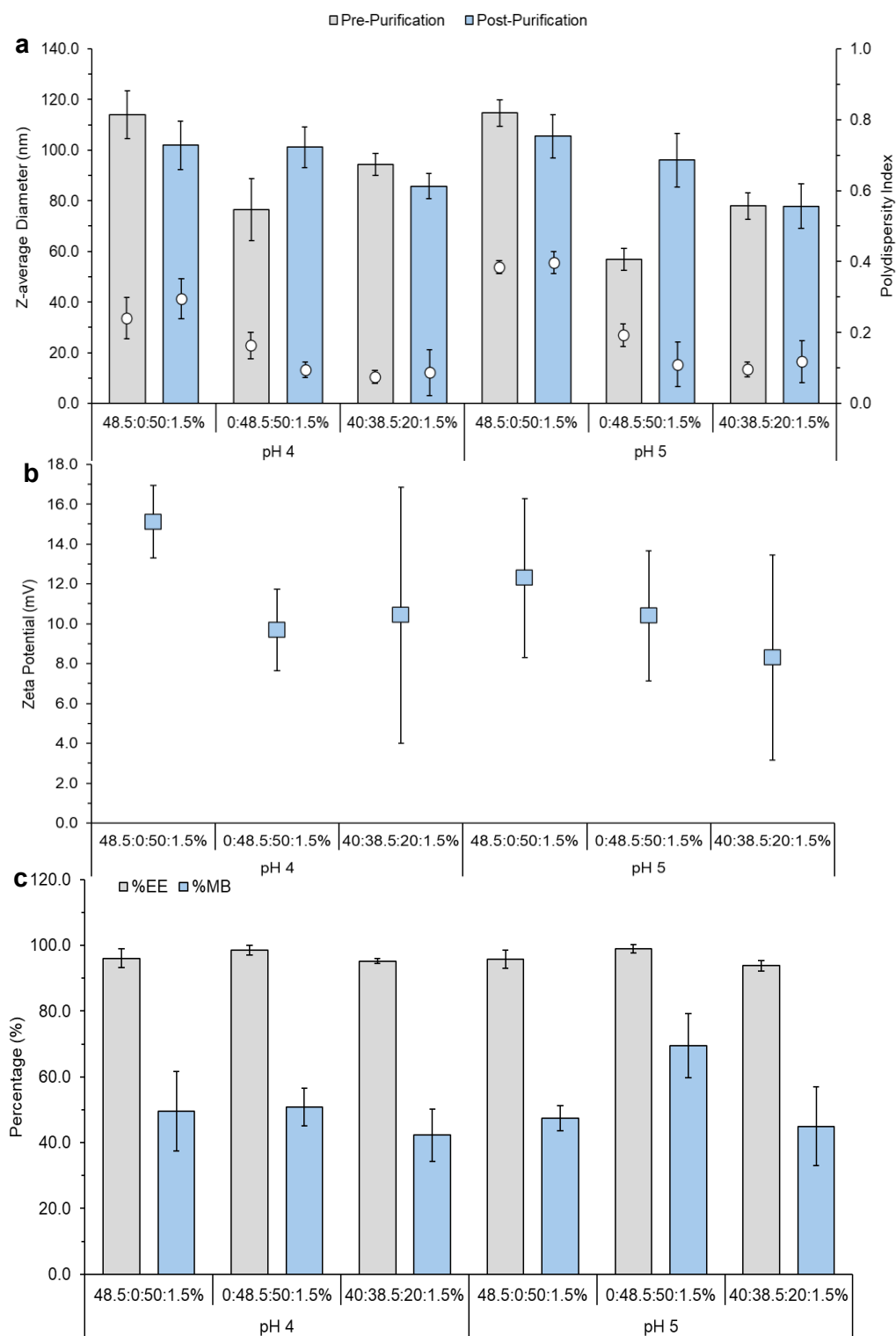


Figure 2-5 The Effect of Aqueous Buffer pH on LNP CQAs. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at molar ratios specified in the graphs encapsulating poly A at a concentration of 0.055 mg/mL purified by spin column. (a) represents the LNP size (nm) and polydispersity index, while (b) shows the zeta potential (mV) and (c) highlights the poly A encapsulation efficiency (%) and poly A recovery (mass balance, (%)). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

2.3.1.4 Purification Buffer Choice

Selecting an appropriate purification buffer often stems from the desired use of the therapeutic. An ideal storage condition for LNPs would be to undergo freeze drying, which would negate the need for any cold chain storage and make global transport easier and cheaper. However, to withstand the freeze-drying process, a cryoprotectant is often required within the buffer to preserve the LNP integrity. These cryoprotectants are often sugars, and it is vital to understand if their inclusions alter the LNP stability or physicochemical properties. Therefore, in this section, the aim was to compare LNPs purified in Tris buffer to LNPs purified in Tris-Sucrose buffer to identify any key differences. The results are shown in Figure 2-6.

The comparison of Tris and Tris-Sucrose buffer was analysed across four LNP formulations with varying molar ratios. Figure 2-6a highlights that for both formulation 1 and formulation 2, purification in Tris-Sucrose resulted in an increase in size of approximately 11 and 20 nm, respectively, whereas formulation 3 experienced a decrease in size of approximately 15 nm in Tris-Sucrose, formulation 4 however, experienced no large changes in size. It can also be noted that only formulation 3 exhibited a change in PDI, with a higher PDI observed in Tris-Sucrose (Figure 2-6a). In all formulations tested, zeta potentials decreased by between 0.4 – 2.6 mV when purified in Tris-Sucrose compared to Tris (Figure 2-6a). Finally, no effects were noted on encapsulation efficiency, but all formulations had increases in payload mass balance between 4-27% when in Tris-Sucrose. Despite these notable differences, no statistically significant changes were witnessed, indicating that LNPs tolerate sucrose well as a cryoprotectant, which could be an advantage during storage and transportation.

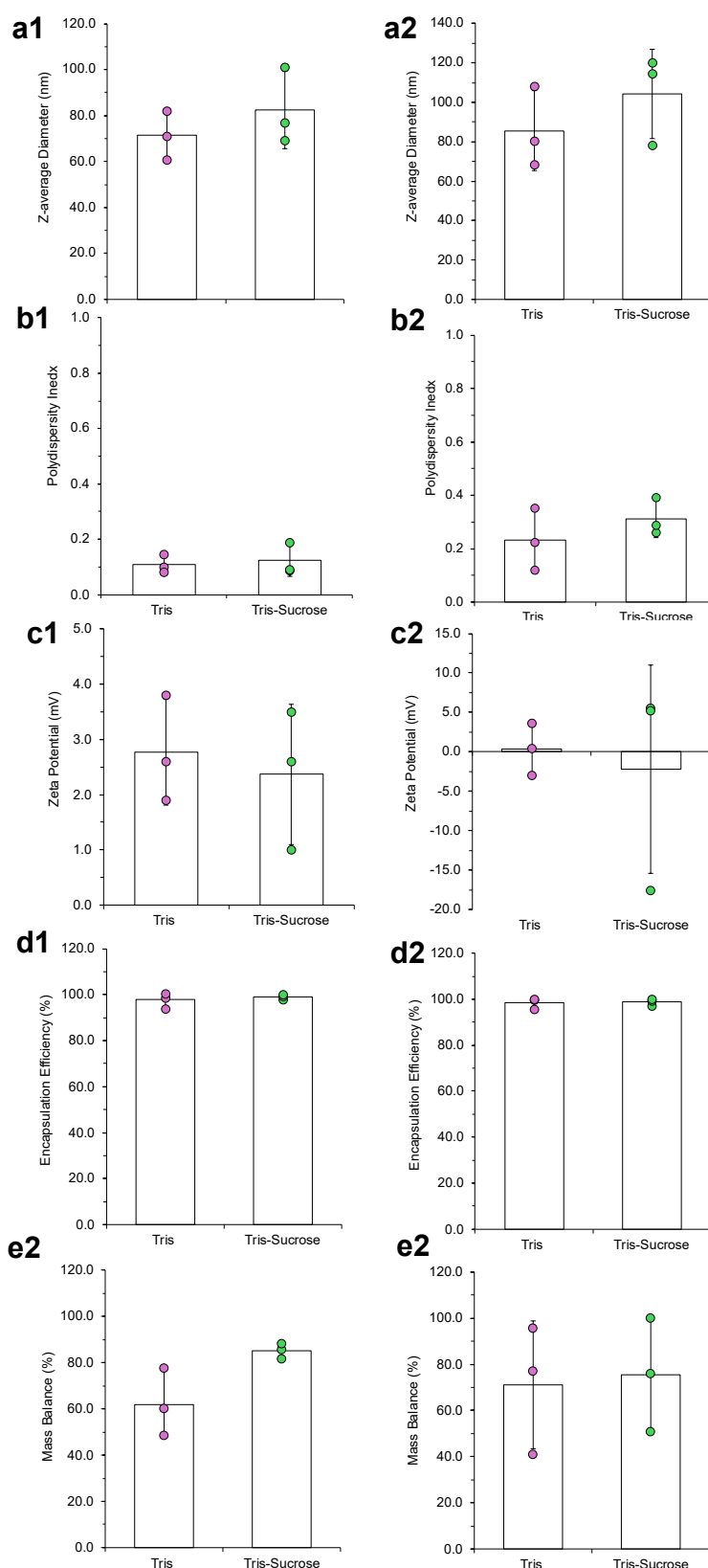


Figure 2-6 The Effect of Purification Buffer. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at molar ratios of 10:38.5:50:1.5% (Formulation 1), 29.5:38:20:2.5% (Formulation 2), 9.5:60:30:0.5% (Formulation 3) or 24.38:40.53:34.17:0.92% (Formulation 4), encapsulating Poly A to a final concentration of 0.055 mg/mL purified by spin. LNPs were purified in either Tris or Tris-Sucrose buffer and analysed for size (nm) (a1-4), polydispersity index (b1-4), zeta potential (mV) (c1-4) and poly A encapsulation efficiency (%) (d1-4) and mass balance (%) (e1-4). Results are representative of the mean $\pm$ standard deviation (SD)(n=3).

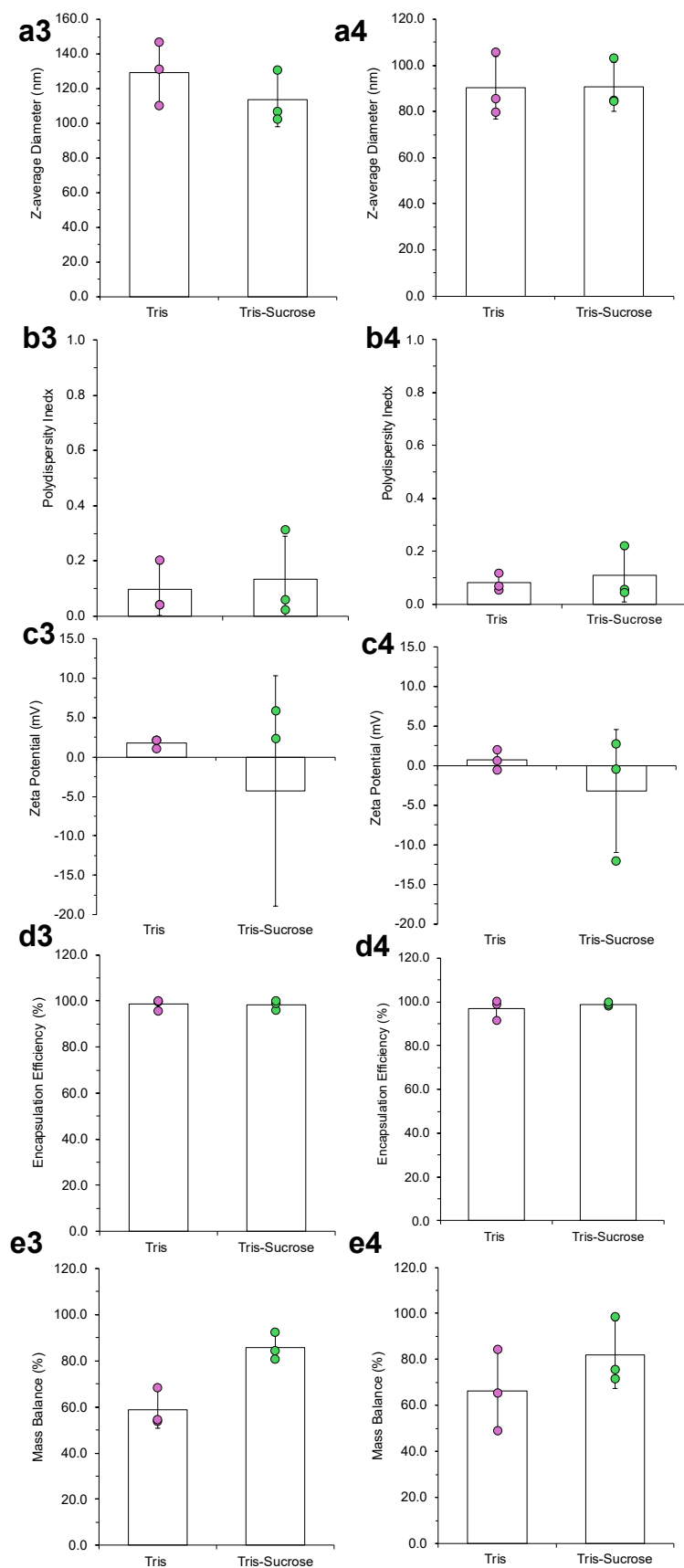


Figure 2-6. Continued.

2.3.1.5 Inclusion of Fluorescent Markers

In addition to monitoring expression levels, an integral part of all nanoparticle efficacy studies will involve monitoring biodistribution, which is normally done by including fluorescent markers. Fluorescent markers can allow whole body tracking of biodistribution patterns as well as aid in identifying off-target effects and delivery kinetics. Labelling of the LNP can be done in a multitude of ways, including lipophilic dyes being incorporated into the bilayer, offering a direct insight into tracking but lacking in the ability to determine functional cargo release, whereas labelling the nucleic acid payload can allow for analysis of intracellular trafficking but often has a negative impact on cargo stability and efficiency. Therefore, the effect of fluorescent labelling on mRNA expression was analysed using three LNP formulations that were manufactured containing either no fluorescent label, the lipophilic label DiR or the hydrophilic label Cy5 conjugated to the nucleic acid payload. The CQAs of the samples are highlighted in Table 2-1, whereas Figure 2-7 shows the bioluminescence and fluorescence output levels.

The data in Table 2-1 highlights that inclusion of lipophilic dyes in the LNP resulted in an increase in size by around 40-50 nm compared to Cy5-labelled or no-labelled LNPs, as well as an increase in heterogeneity indicated by an increased PDI >0.2, with minimal effects on LNP charge. In addition, DiR-labelled LNPs also showed decreased mRNA mass balance compared to Cy5- or no-labelled LNPs (Table 2-1). Furthermore, inclusion of dyes had no effect on mRNA integrity as shown in Figure 2-8.

Following treatment in BALB/c mice, the Cy5 and DiR fluorescence signals alongside bioluminescence of the LNPs were monitored 3 and 6 hours post-injection. Figure 2-7a-c highlight the signal quantified from the injection site, while Figure 2-7d-f highlights signals quantified from the liver and spleen following ex vivo imaging.

While it can be seen that DiR- and no-labelled LNPs also produced a background signal of Cy5 fluorescence, the Cy5-containing LNP had significantly higher ($p > 0.001$) signal at both 3 and 6 hours, indicating a significant proportion of the LNPs remained at the injection site (Figure 2-7a). This is further evidenced by ex vivo imaging, which showed no significant differences in Cy5 fluorescence in either the liver or the spleen across all samples (Figure 2-7d).

Likewise, DiR-labelled LNPs carry a significantly increased signal at the injection site ($p < 0.001$) at all time points compared to that of Cy5- or no-labelled LNPs (Figure 2-7b), agreeing with the Cy5 fluorescence that a high proportion of LNPs remain at the injection site. However, ex vivo imaging of the liver and spleen for DiR fluorescence did highlight significantly increased DiR levels in the liver ($p < 0.001$), indicating that a portion of LNPs may have travelled throughout the body (Figure 2-7e). However, it is possible this was not picked up *in vivo* due to low tissue penetration.

Finally, when looking at protein expression as a function of bioluminescence, it can be seen that both fluorescent labels had a negative impact on protein expression at all time points (Figure 2-7c). At the 3 hour time point, the no-label LNP had significantly higher bioluminescence compared to the DiR-labelled LNP with significantly higher expression seen against Cy5-labelled LNPs at the 6 hour time point ($p < 0.001$) (Figure 2-7c). Additionally, this effect was also evident ex vivo, with only the DiR-labelled LNPs liver expression having no statistical difference compared to no-label LNPs (Figure 2-7f). However, it should be noted that the decreased expression has not been directly linked to fluorescent label use hence interference of the fluorophore cannot be excluded.

Overall, this indicates that when using fluorescent labels on LNPs, care should be taken as to the method of labelling alongside ensuring accurate controls that account for the effect of the fluorescent label

Table 2-1 Critical Quality Attributes of LNPs manufactured including varied Fluorescent Dyes. LNPs were manufactured on the AXF-Mini Mixer and consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ with or without DiR and encapsulating either Firefly Luciferase mRNA or Cy5-Labelled Firefly Luciferase mRNA and concentrated to a final concentration of 0.16 mg/mL. Results are representative of one independent repeat as the mean $\pm$ standard deviation.

Fluorescent Label	Z-average Diameter (nm)	Polydispersity Index	Zeta Potential (mV)	Encapsulation Efficiency (%)	Mass Balance (%)
Cy5	108.7 $\pm$ 1.9	0.077 $\pm$ 0.007	8.2 $\pm$ 1.7	91.2	77.4
No Label	117.8 $\pm$ 0.4	0.092 $\pm$ 0.014	10.4 $\pm$ 1.4	91.9	88.0
DiR	157.1 $\pm$ 4.6	0.242 $\pm$ 0.026	12.1 $\pm$ 1.3	96.3	46.9

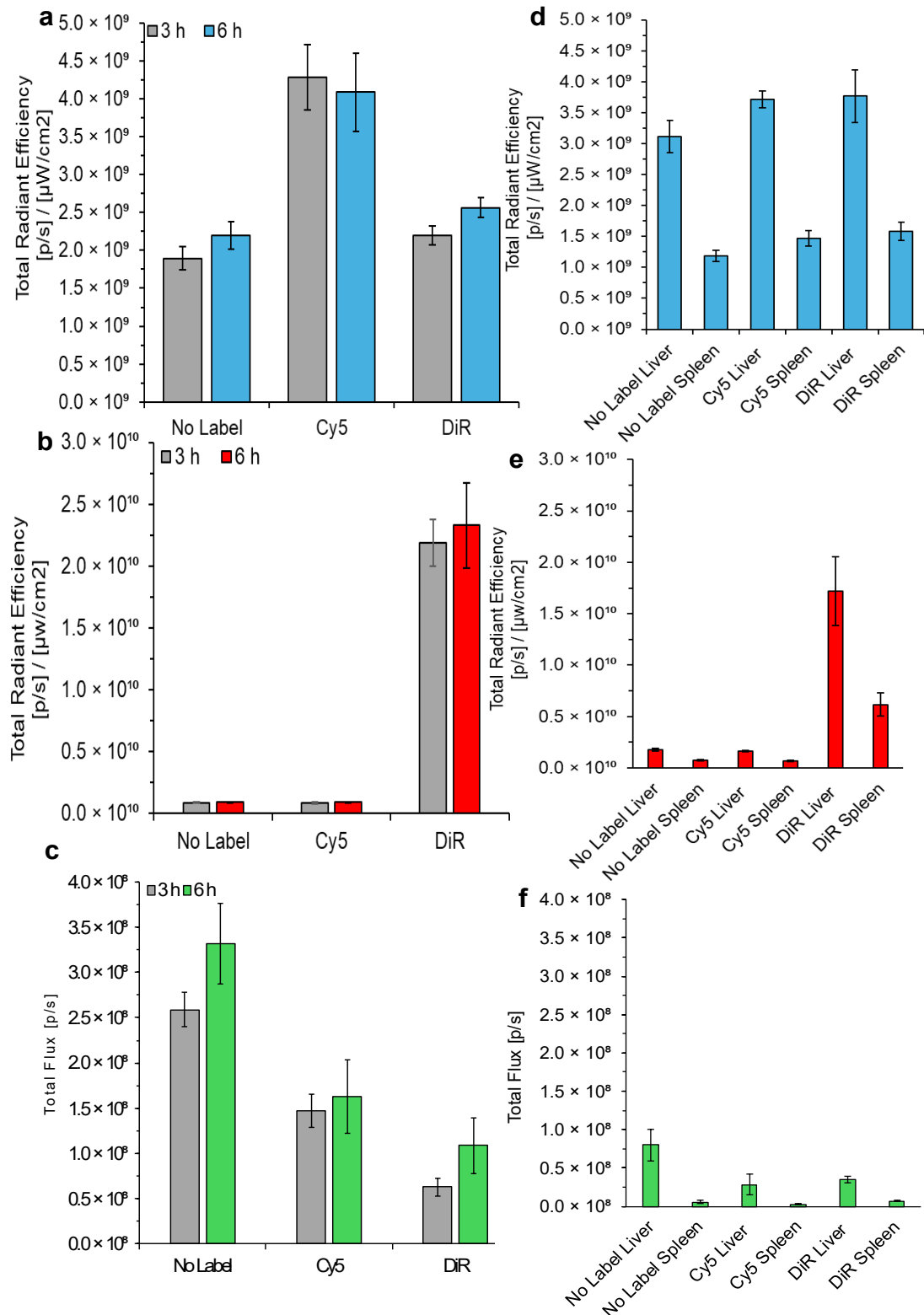


Figure 2-7 *In vivo* performance of mRNA-LNPs manufactured with either no fluorescent label, DiR or Cy5-tagged Fluc. BALB/c mice were intramuscularly injected with 3.5 μ g/mL mRNA per leg (7 μ g total dose) and, 3 h and 6 h post injection total Cy5 (a), DiR (b) and Bioluminescence (c) signals were captured for all mice. At the 6 h time point, mice were also culled, and the liver and spleen were removed for ex vivo imaging under the Cy5 (d), DiR (e), and Bioluminescence (f) filters. All signals were quantified using Living Image 4.0 software with regions of interest encompassing the injection site and liver/spleen. Results are representative of the mean $\pm$ standard error of the mean (SEM) (n=6 for injection site (a)) and (n=3 for liver/spleen (b)).

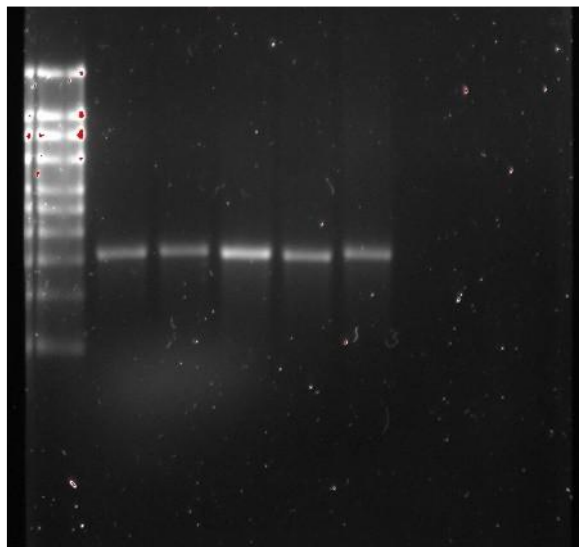


Figure 2-8 mRNA Integrity of LNPs with no-label, a Cy5-label or a DiR-label. Integrity was monitored using gel electrophoresis and samples were included as follows: RNA Millennium Marker (Lane 1), Firefly Luciferase mRNA Control (Lane 2), Cy5-Labelled Firefly Luciferase mRNA Control (Lane 3) and mRNA extracted from Cy5-Labelled LNPs (Lane 4), No-Label LNPs (Lane 5) and DiR-Labelled LNPs (Lane 6). Gel was imaged using Bio-Rad EZ Doc Gel Imager and analysed using Bio-Rad Image Lab v6.1.

2.3.2 The Effect of Process Parameters

Following on from formulation parameters, the next vital process parameters are often mechanistic and can be termed critical process parameters (CPPs). These independent CPPs will include manufacturing parameters such as microfluidic mixer choice, manufacturing speeds and ratios, and small variations in these variables can significantly impact formulation CQAs and function. Therefore, it is critical to determine the correct CPPs to enhance reproducibility and ensure robustness within the methodology. In this section, the effect of total flow rates (TFR) and flow rate ratios (FRRs) as well as microfluidic mixer choice on resulting LNPs and their CQAs and efficacy was tested. Additionally, this section will investigate how head and tail cut offs in manufacture can ensure accurate results with high repeatability.

2.3.2.1 How Adjusting the TFRs and FRRs can Allow for Accurate Control of LNP Characteristics

Two of the most vital CPPs considered when manufacturing LNPs are the speed at which the two phases are mixed (Total Flow Rate (TFR)), as well as the ratio at

which they are mixed (Flow Rate Ratio (FRR)), with previous research establishing these CPPs as methods of controlling particle size. In this results section, the effect of both FRR and TFR will be systematically reviewed first with a cationic-LNP formulation using a staggered herring bone mixer (Figure 2-9), next in three varied mixing modalities using an ionizable-LNP formulation (Figure 2-10), and lastly employing the same ionizable-LNP formulation on an impinged jet mixer (Figure 2-11), and finally determining if these changes result in any efficacy differences in an *in vivo* and *in vitro* model (Figure 2-12). By conducting this analysis, the results highlighted that the varied effect of both CPPs and as well as demonstrating how different machines can increase variability.

Figure 2-9 highlights the CQAs achieved when DOTAP-LNPs were manufactured on the SHM (NanoAssemblr®, Cytiva) at TFRs of 10 or 20 mL/min and FRR of 2:1, 3:1 and 4:1 (aqueous:lipid). No significant changes in size ($p>0.05$) with all test conditions resulting in LNPs between 74-114 nm post-purification. Significant differences ($p<0.001$) were only seen for LNPs manufactured at 2:1 20 mL/min and LNPs manufactured at 4:1 20 mL/min, with the latter having increased PDI ~ 0.29 (Figure 2-9a). No significant differences were seen for the zeta potential of LNPs with slight positive readings between 1.4-4.3 mV recorded (Figure 2-9b). For LNPs manufactured at 10 mL/min, encapsulation was significantly improved when the FRR was 3:1 or 4:1 ($p<0.001$), while for LNPs manufactured at 20 mL/min, a significant ($p<0.05$) increase in encapsulation was only seen for LNPs manufactured at 3:1 (Figure 2-9c). However, changes to these CPPs resulted in no significant differences amongst payload mass balance ($p>0.05$).

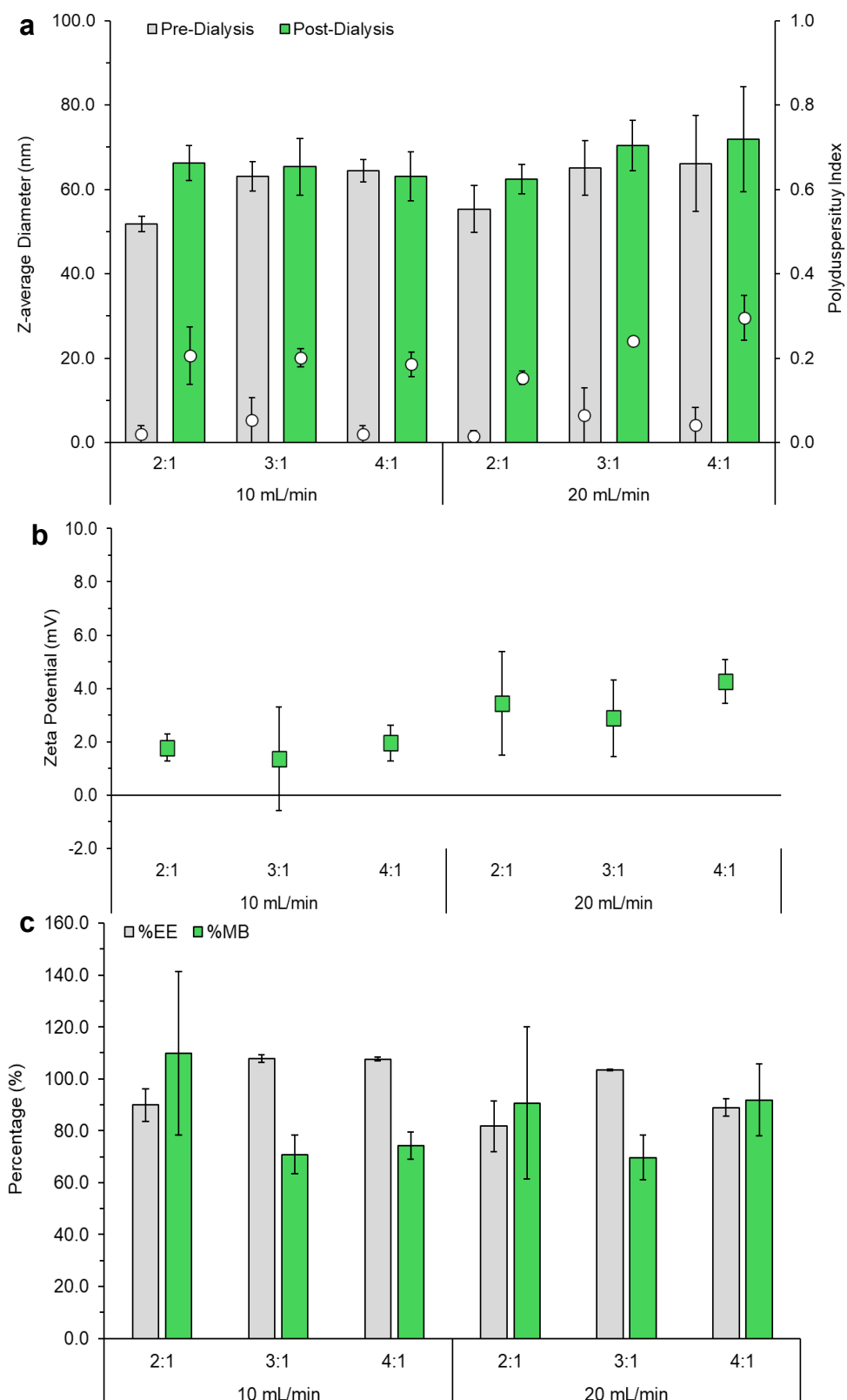


Figure 2-9 The Effect of TFR and FRR on DOTAP LNPs. LNP consisted of DSPC:cholesterol:DOTAP:DMG-PEG₂₀₀₀ encapsulating poly A to a final concentration of 0.055 mg/mL manufacture on the SHM using NanoAssemblr© and purified by dialysis. (a) represents z-average diameter (nm) and polydispersity index while (b) represents zeta potential (mV) and (c) shows poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

While Figure 2-9 highlighted the role which FRR and TFR play at research scale manufacturing settings, the NanoAssemblr© is limited to an upper TFR range of 20 mL/min and a sample volume of 15 mL. This limits its applicability to larger-scale manufacture.

To further investigate the importance of TFR, three microfluidic mixing methods were utilised: a T-junction mixer, a Confined Impingement Jet mixer or the AXF-mini mixer. Each system operated via an automated two-pump system controlled by external syringe pump software. To allow analysis of one standard TFR 15 mL/min and two high TFRs 30 and 60 mL/min. Thereby extending our knowledge of how TFR affects SM-102 LNPs and how this could be more applicable to large scale manufacture.

Figure 2-10a demonstrates that for all three mixing methods, an increase from 15 to 30 mL/min causes a decrease in size. A further increase to 60 mL/min causes a further increase in size, both pre- and post-purification, with all sizes lying between 74-124 nm. Additionally, for both the T-junction mixer and Impingement Jet mixer, PDI generally increases when TFR increases, whilst the AXF-Mini remains largely unaffected. No changes in size or homogeneity were deemed to be statistically significant ($p > 0.05$). Furthermore, only the T-junction mixer had significantly decreased zeta potential when LNPs were manufactured at 15 or 30 mL/min ($p < 0.05$). Finally, increasing TFR had no significant adverse effect on poly A encapsulation or mass balance.

Overall, this data highlights that the standard SM-102 LNP is able to withstand faster manufacturing speeds, which can in turn decrease manufacturing timescales and cost and offer enhanced batch to batch repeatability.

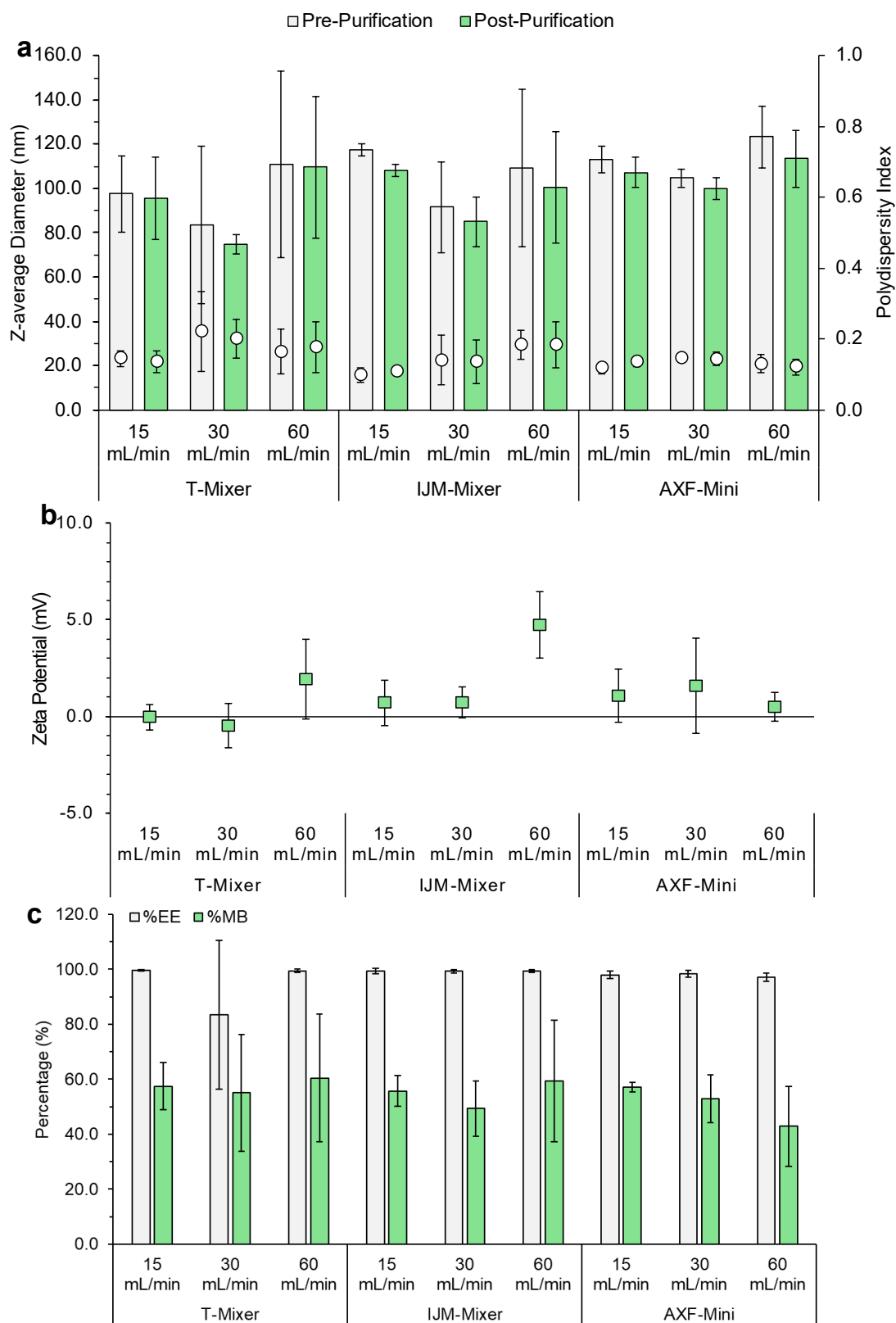


Figure 2-10 The Effect of Increasing TFR on SM-102 LNPs. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating poly A to a final concentration of 0.055 mg/mL and purified using dialysis. (a) represents the LNP Z-average diameter (nm) and polydispersity index while (b) represents the zeta potential (mV) and (c) represents the poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

Furthermore, to establish the effect of a wider range of lower TFRs, an impingement jet style microfluidic mixer was employed (Nova Benchtop, Helix Biotech) with interchangeable mixer sizes (sizes 0, 1, or 2) to enable continuous increases in TFR. Utilising this, SM-102 LNPs were manufactured across a range of TFRs from 6-35 mL/min, and the results are shown in Figure 2-11. Furthermore, to ensure TFR had no effect on efficacy, TFRs 8, 15 and 32 were chosen to manufacture SM-102 LNPs encapsulating Firefly Luciferase mRNA, allowing for *in vitro* and *in vivo* protein expression to be analysed with results shown in Figures 2-12 and 2-13.

Figure 2-11 highlighted how increasing the TFR ranges across the three mixer sizes had no significant effect ($p>0.05$) on any of the CQAs measured. All LNPs were between 55-69 nm pre- and post-purification, with PDI consistently <0.134 , zeta potentials between -1 and 1 mV, high poly A encapsulation $>99\%$, and mass balance $>63\%$. This interchangeability is beneficial for bench-scale research as it enhances the reproducibility and reliability of results.

Following initial analysis, LNPs were re-formulated at TFR 8, 15 and 32 but this time encapsulating Fluc or Green Lantern mRNA. Table 2-2 highlights the CQAs of the LNPs, showing that overall mRNA-LNPs show increased sizes compared to poly A-LNPs, ranging between 110-137 nm with PDI <0.12 and zeta potentials between -2.5 and 2.5 mV. Additionally, mRNA encapsulation is $>93\%$ while mass balance is $>69\%$. All LNPs were deemed to be within acceptable limits and were employed in the analysis of *in vitro* and *in vivo* protein expression.

Figures 2-12 and 2-13 show the corresponding *in vitro* and *in vivo* data. Highlighting that the formulations show no cell toxicity (Figure 2-12b) and are identical *in vitro* protein expression profiles across all concentrations/mRNAs tested (Figure 2-12a and Figure 2-13). Furthermore, Figure 2-12c/d demonstrates that all tested LNPs had similar *in vivo* protein expression levels and

biodistribution at both the injection site and the liver regions of interest. Together, this data emphasises that TFR does not affect the efficacy of formulations offering a more flexible manufacturing model.

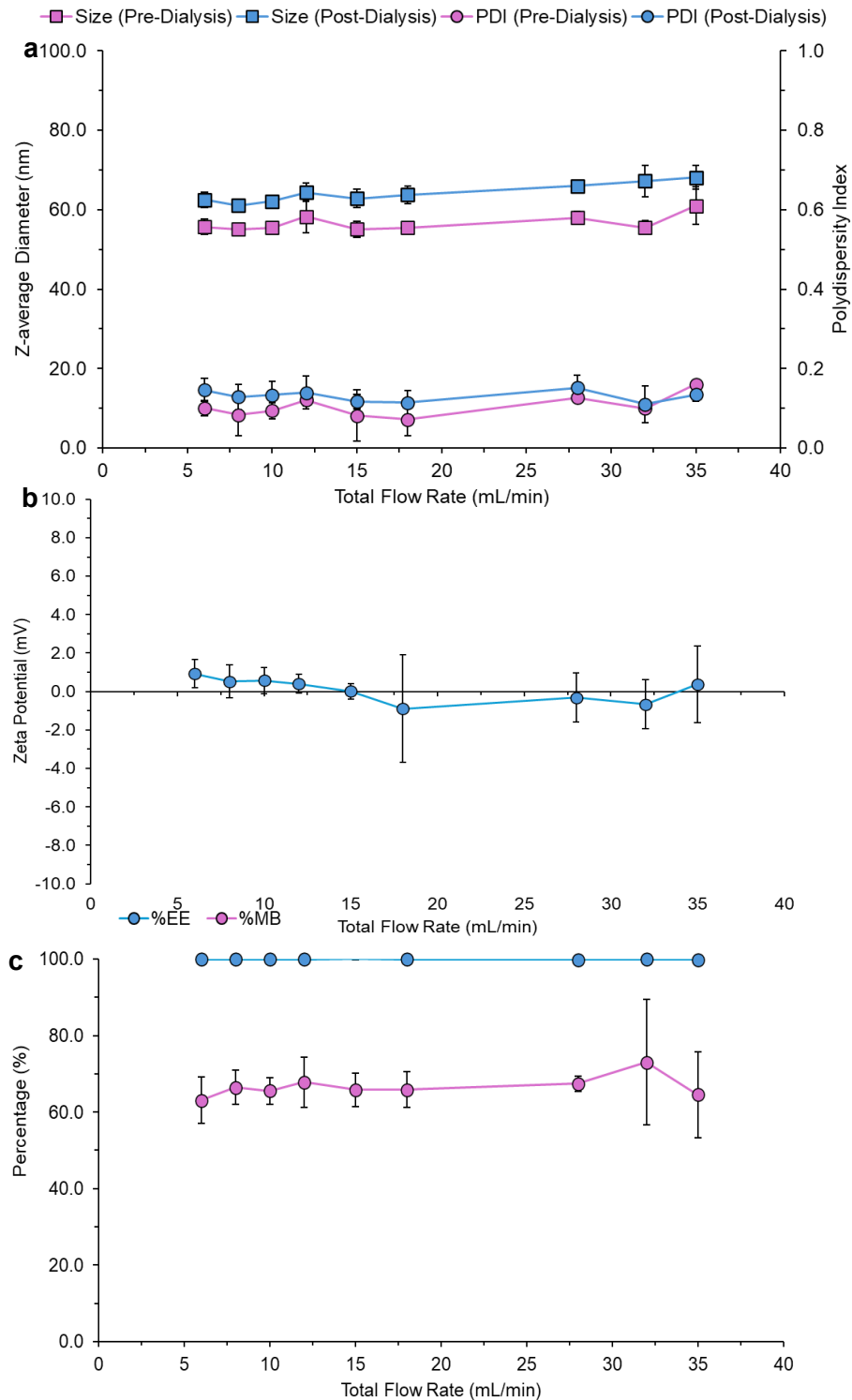


Figure 2-11 The Effect of Continuously Increasing TFR on SM-102 LNPs. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating poly A to a final concentration of 0.025 mg/mL and purified using dialysis. TFRs 6-10 mL/min were manufactured on the size 0, 12-18 mL/min were manufactured on size 1 and 28-35 mL/min were manufactured on size 2 mixers. (a) represents the LNP Z-average diameter (nm) and polydispersity index while (b) represents the zeta potential (mV) and (c) represents the poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

Table 2-2 Critical Quality Attributes of LNPs manufacturing at varied TFRs. LNPs were manufactured on the Nova Benchtop® and consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating either Firefly Luciferase mRNA or GrL mRNA encoding for GFP to a final concentration of 0.025 mg/mL. Results are representative of mean $\pm$ standard deviation (SD) (n=3).

mRNA	TFR (mL/min)	Z- average Diameter (nm)	Polydispersity Index	Zeta Potential (mV)	Encapsulation Efficiency (%)	Mass Balance (%)
Fluc	8	110.2 $\pm$ 18.8	0.101 $\pm$ 0.024	2.3 $\pm$ 3.6	94.9 $\pm$ 2.8	79.7 $\pm$ 11.5
	15	115.6 $\pm$ 22.0	0.107 $\pm$ 0.027	0.4 $\pm$ 2.0	94.9 $\pm$ 3.1	79.8 $\pm$ 6.1
	32	116.9 $\pm$ 19.6	0.101 $\pm$ 0.031	0.8 $\pm$ 3.3	93.9 $\pm$ 3.0	75.0 $\pm$ 10.6
GrL	8	130.9 $\pm$ 14.9	0.111 $\pm$ 0.046	-1.1 $\pm$ 0.9	93.7 $\pm$ 4.5	82.0 $\pm$ 3.7
	15	136.7 $\pm$ 18.3	0.093 $\pm$ 0.050	-2.3 $\pm$ 0.9	94.3 $\pm$ 3.3	70.0 $\pm$ 7.6
	32	127.0 $\pm$ 9.4	0.075 $\pm$ 0.031	-1.7 $\pm$ 1.1	95.2 $\pm$ 1.1	69.3 $\pm$ 4.6

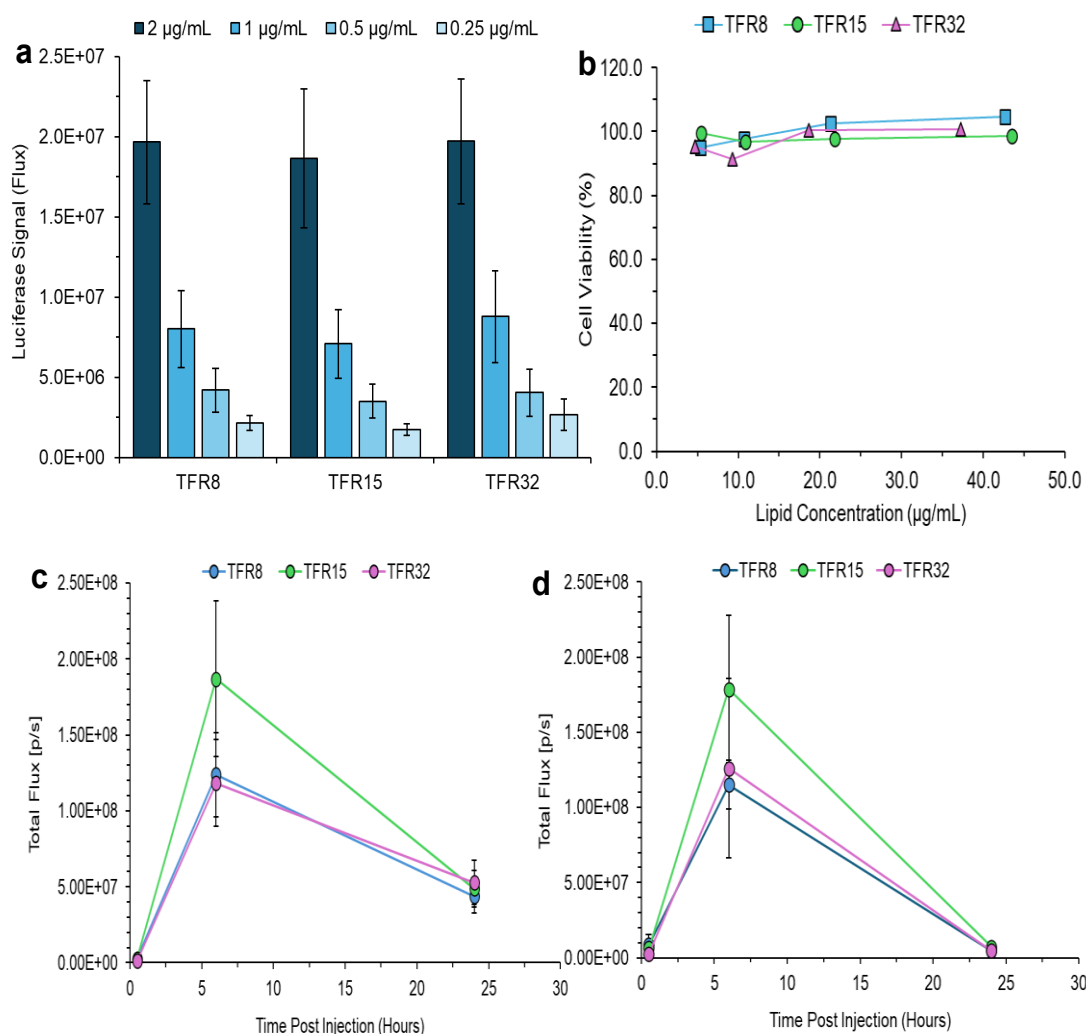


Figure 2-12 The In Vitro and In Vivo Performance of LNPs manufactured at Varying TFRs. Fluc-LNPs from Table 2 were used to treat HEK-293 cells at mRNA concentrations of 2-0.25 µg/mL for analysis of (a) in vitro protein expression and (b) cell viability 48 hours post-treatment. Fluc-LNPs were I.M injected into BALB/c mice at a total concentration of 2 µg mRNA and protein expression monitored up to 24 hours for both injection site (c) and liver (d). Results are representative of the mean $\pm$ standard error of the mean (SEM) (n=3).

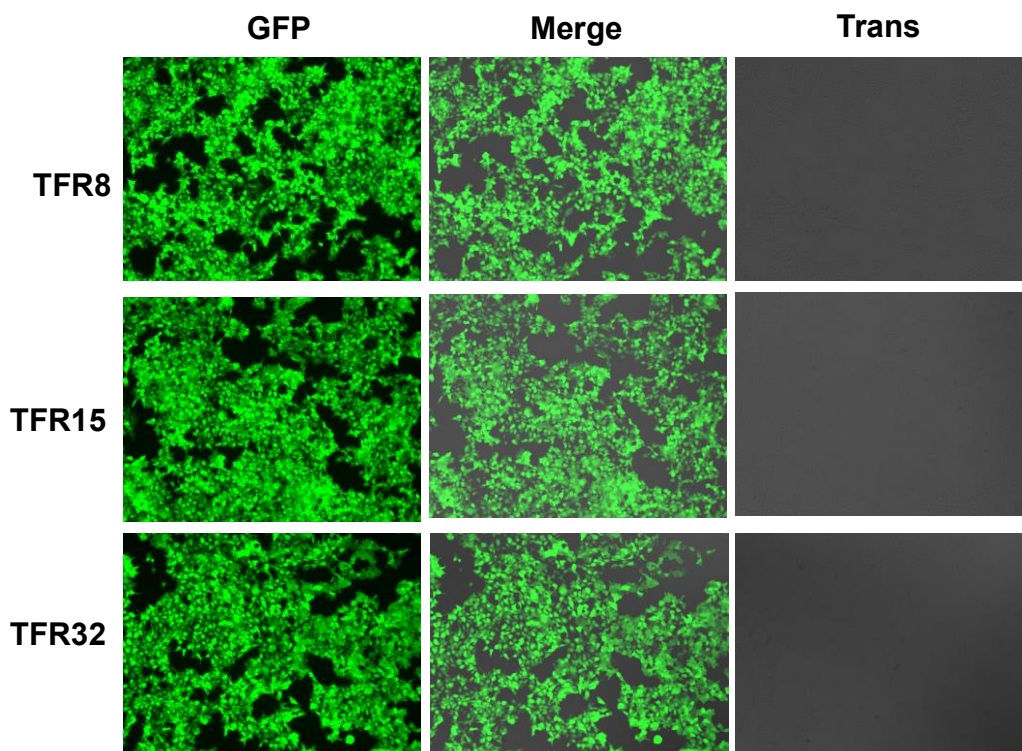


Figure 2-13 Representative Images of Green Fluorescent Protein Expression in HEK-293 cells following GrL-LNP treatment at a concentration of 2 $\mu\text{g}/\text{mL}$ for 48 hours. Images were taken on the EVOS 5000 microscope using the GFP and Trans bulbs at a magnification of 10x, scale bar = 300 μm .

To conclude the analysis of CPPs, FRR was reviewed on the Nova Benchtop (Helix Biotech) at ratios between 1:1 and 5:1 (aqueous:lipid) and the results are represented in Figure 2-14.

Figure 2-14a highlights that pre- and post-purification, a ratio of 1:1 results in significantly larger and more unstable LNPs, indicated by variable sizes >300 nm compared to the remaining ratios ($p < 0.001$), with sizes decreasing as ratios are increased and becoming steady at a ratio of 2:1. Similar trends can also be seen for PDI (Figure 2-14a). Not significant differences were seen for the zeta potentials ($p > 0.05$) with all LNPs being considered near neutral, ranging between -5 and 1 mV (Figure 2-14b). Additionally, encapsulation efficiency showed no clear effects, however, significantly increased poly A mass balance was seen in the 1.5:1 ratio compared to the 1:1 ratio ($p > 0.05$) followed by a significant decrease in poly A mass balance when ratios equalled 5:1 ($p < 0.01$) (Figure 2-14c).

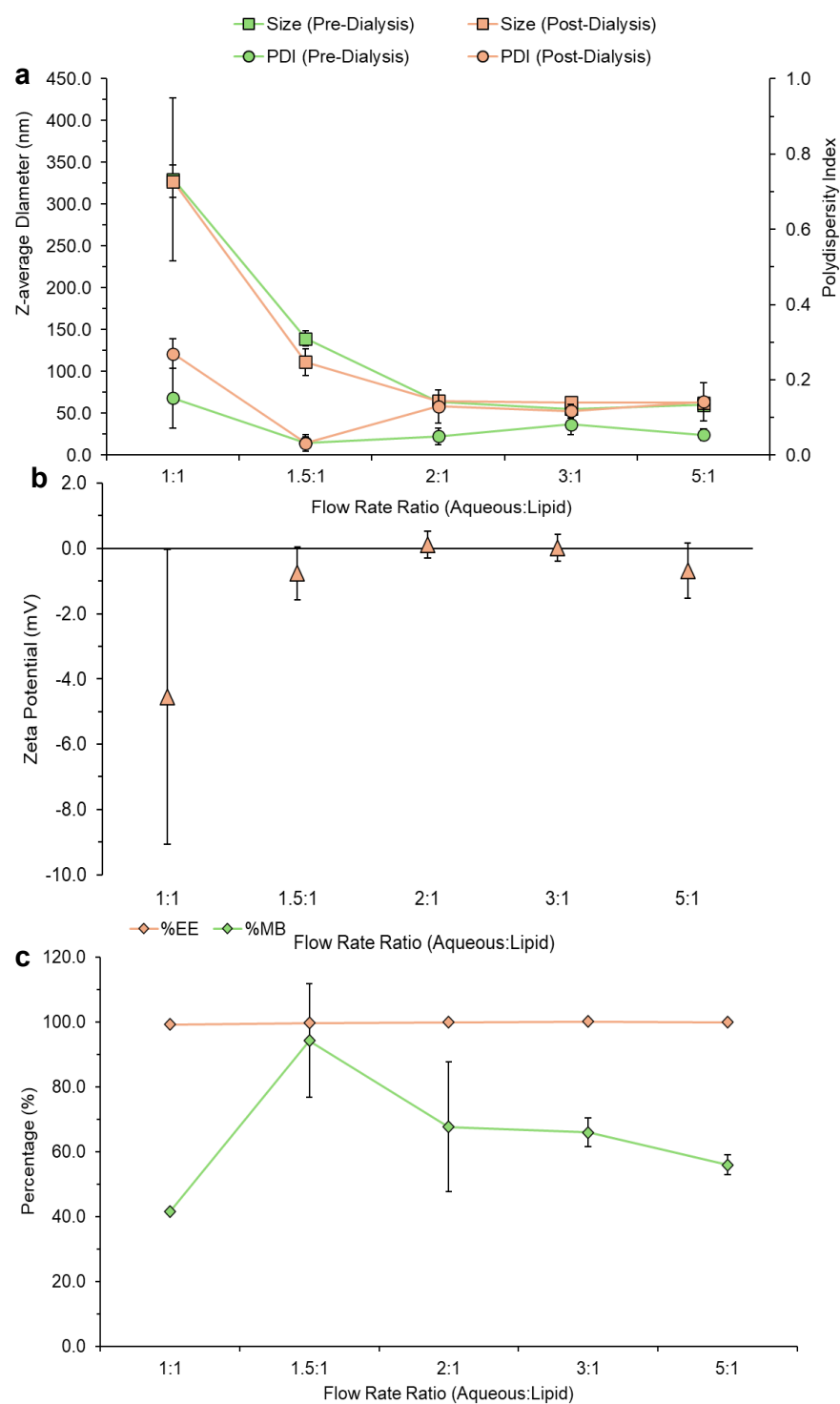


Figure 2-14 The Effect of FRR on SM-102-LNPs. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at a molar ratio of 10:38.5:50:1.5% encapsulating poly A to a final concentration of 0.025 mg/mL and purified by dialysis. (a) represents the z-average diameter (nm) and polydispersity index, (b) represents the zeta potential (mV) and (c) represents poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

2.3.2.3 The Effect of Mixer Geometry and Purification Choice

The geometry of the chosen microfluidic mixer heavily governs the efficacy of the mixing by shaping the fluid dynamics and thus influencing key factors such as mixing kinetics, shear force profiles, equilibration times, and, consequently, assembly efficacy for LNPs. Furthermore, after microfluidics, the LNPs will need to undergo a purification step to remove residual ethanol. However, the purification steps themselves may induce colloidal instability, reducing payload encapsulation and affecting the final drug product. To consider how LNPs assemble when manufactured on different mixers, a staggered herringbone mixer (NanoAssemblr, Cytiva), Toroidal mixer (Ignite NanoAssemblr, Cytiva) and the Impingement Jet mixer (Nova, Helix Biotech) were employed to manufacture the LNPs, which were then subjected to two purification methods: dialysis and spin column chromatography. The resulting LNP CQAs were analysed, and the results are shown in Figure 2-15.

Figure 2-15 highlights the effect of dialysis and spin column purification methods on SM-102 LNPs. With dialysis resulting in a non-significant increase ($p>0.05$) of between 8-12 nm compared to pre-purification LNPs for all mixers, whereas spin column purification resulted in a significant increase of between 28-41 nm ($p<0.001$) in size compared to the pre-purification LNPs (Figure 2-15a). Additionally, only the LNPs manufactured on the Ignite and purified using spin column showed a significantly increased zeta potential equal to ~21 mV (Figure 2-15b). On the contrary, the choice of purification did not affect poly A encapsulation efficiency or mass balance ($p>0.05$) (Figure 2-15c). Overall, the mixer choice showed no significant differences in the measured characteristics, indicating that research-scale manufacturing methods are generally interchangeable, offering control over CQAs and enhanced batch-to-batch repeatability.

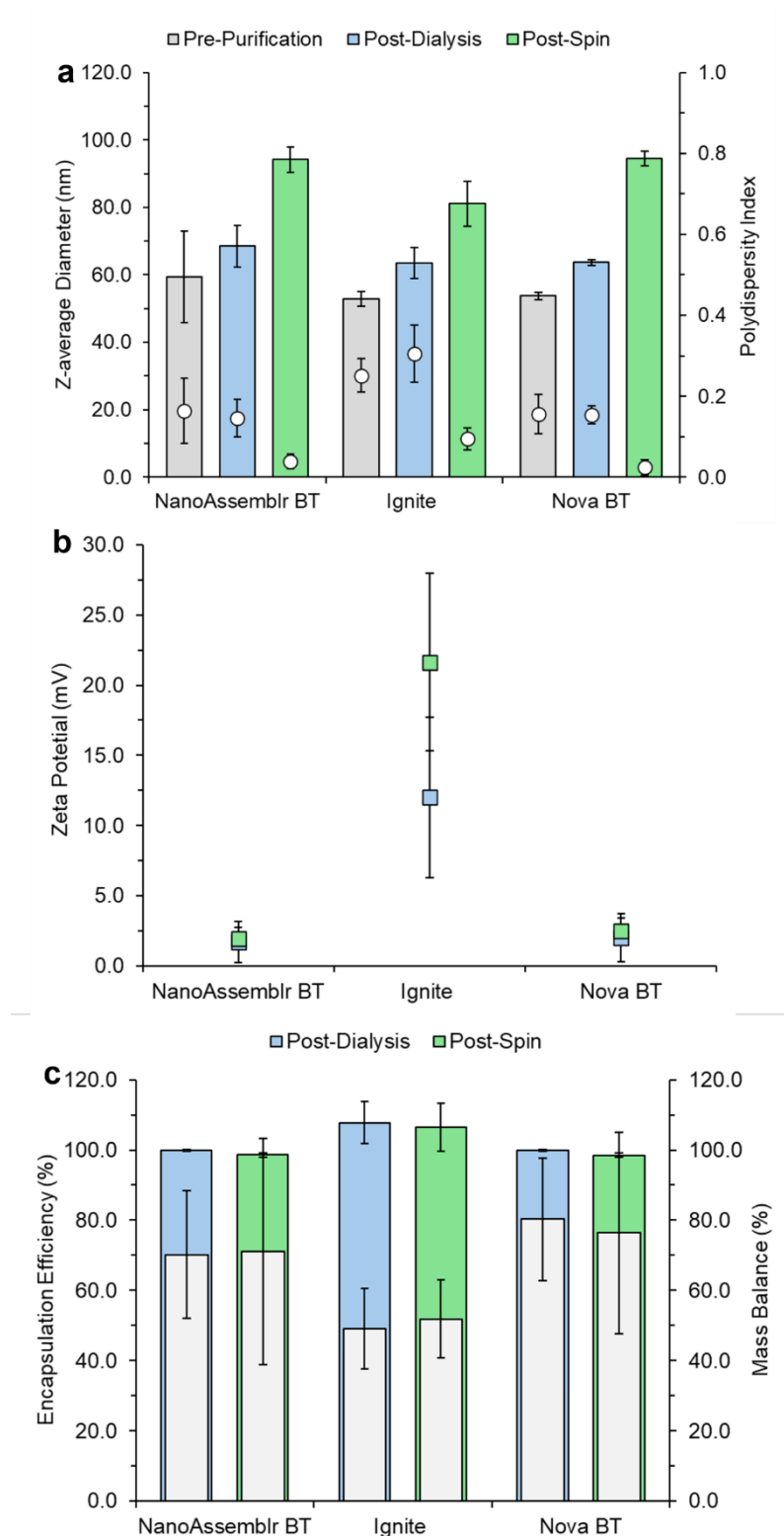


Figure 2-15 The Effect of Mixer Choice and Purification. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at a molar ratio of 10:38.5:50:1.5% encapsulating poly A to a final concentration of 0.055 mg/mL. (a) represents the z-average diameter (nm) and polydispersity index, (b) represents the zeta potential (mV) and (c) represents poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=3).

2.3.2.4 Determining the Most Efficient Wastage

Often under reported in literature, a key determinant of machine choice is often the efficient wastage needed to allow the mixing to fully equilibrate offering precise LNP formulation even at small scale research (<1 mL). Determining the correct wastage for a machine is often driven by several factors including tubing length and dead volume. At research scale, materials are costly and generally available in limited quantities, therefore it is essential to use minimal material to get the maximum response. Therefore, a pilot study was undertaken to analyse wastage volume across several mixing modalities to determine how low head- and toe-cut-offs can be set before the effects of these changes are observed in the CQAs of the LNPs.

Figure 2-16 highlights the CQA results from LNPs manufactured on either the T-junction mixer, confined impingement jet mixer or AXF-mini mixer with wastages of 0, 250 and 500 μ L. For both the T-mixer and IJM-mixer, pre-purification sizes/PDIs were relatively unaffected by the changes to the wastage amounts, with a wastage of 250 μ L resulting in the largest sizes for both mixers. On the other hand, the AXF-mini can maintain a consistent size and homogeneity with wastage of 250 or 500 μ L but becomes large and unstable when 0 μ L wastage is selected (Figure 2-16a). Differences in tolerability are likely due to the mixing methods employed with the AXF-mini – longer equilibration times may be needed to allow effective crossflow of the lipid phase, compared to the instant phase mixing of the T-mixer and IJM-mixer. The 250 μ L waste LNPs in both the T-mixer and IJM-mixer also resulted in increased zeta potentials, but these were still maintained within a near neutral level (Figure 2-16b). The largest effect of wastage is seen when poly A encapsulation efficiency and mass balance were recorded (Figure 2-16c). Overall, the T-mixer was larger and unaffected compared to the IJM-mixer and AXF-mixer, both of which achieve acceptable encapsulation levels only when wastage is 500 μ L (Figure 2-16c). Together, these results highlight that

small changes in wastage can have extensive effects on CQAs, leading to lower LNP efficacy and formulation instability.

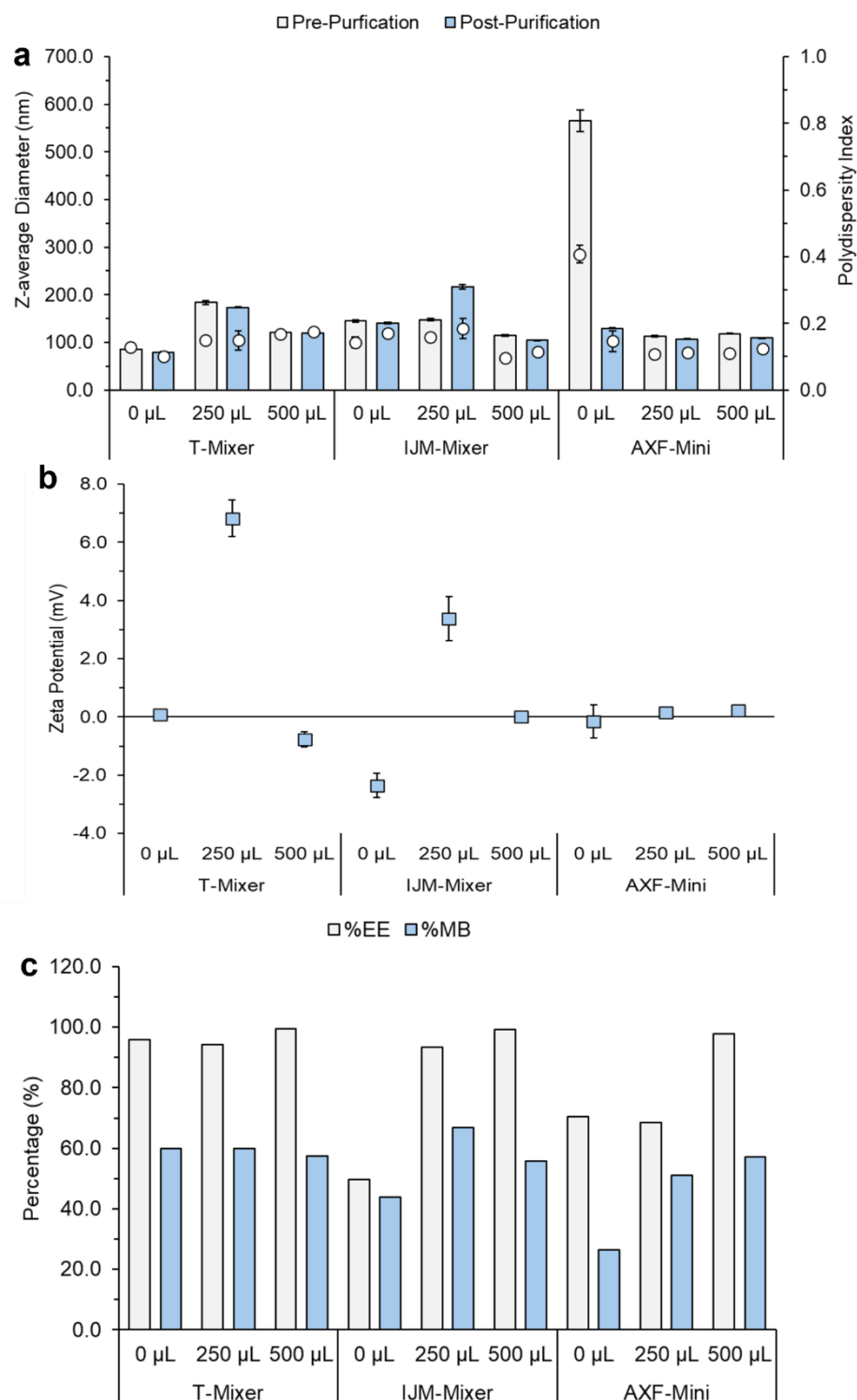


Figure 2-16 The Effect of Wastage on Low-Cost Microfluidic Mixers. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at a molar ratio of 10:38.5:50:1.5% encapsulating poly A to a final concentration of 0.055 mg/mL. (a) represents the z-average diameter (nm) and polydispersity index, (b) represents the zeta potential (mV) and (c) represents poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) (n=1).

While Figure 2-16 highlights the importance of one-off wastage analysis, it lacks detailed knowledge of the levels between tested volumes, as well as of how wastage between samples can lead to cumulative effects. Therefore, we next manufactured LNPs on the Nova Benchtop (Helix, Biotech) at waste volumes ranging from 0 to 500 μL , either with no wash between LNPs or with an ethanol/water wash between. The results of the gathered CQAs are shown in Figure 2-17.

When LNPs are manufactured without a wash between wastage conditions, sizes appear to be maintained at ~ 80 nm, with PDI < 0.2 both pre- and post-purification, indicating that wastage is not a limiting factor (Figure 2-17a). However, without a wash in between LNPs, there is likely to be spillover from previous samples, which could lead to contamination. Hence, when the ethanol/water wash was incorporated, a large increase in sizes was seen when wastage was ≤ 200 μL (Figure 2-17a) with sizes being > 90 nm, indicating that LNP mixing has not yet equalled equilibrium and collecting samples within this range can be detrimental to colloidal stability and encapsulation efficiency. No major effects were seen on post-purification zeta potential (Figure 2-17b). However, poly A mass balance was affected with an increase poly A mass balance seen at higher wastage volumes when a wash is incorporated, with $\sim 65\%$ mass balance at 500 μL compared to $\sim 40\%$ at 0 μL , while LNPs made with no wash maintained steady mass balances between 55-60% (Figure 2-17c). Encapsulation efficiency remained unaffected (Figure 2-17c).

Overall, this indicates that not only is wastage machine specific, but it is also operating procedure specific and best practice would always be to consider this an optimisable CPP.

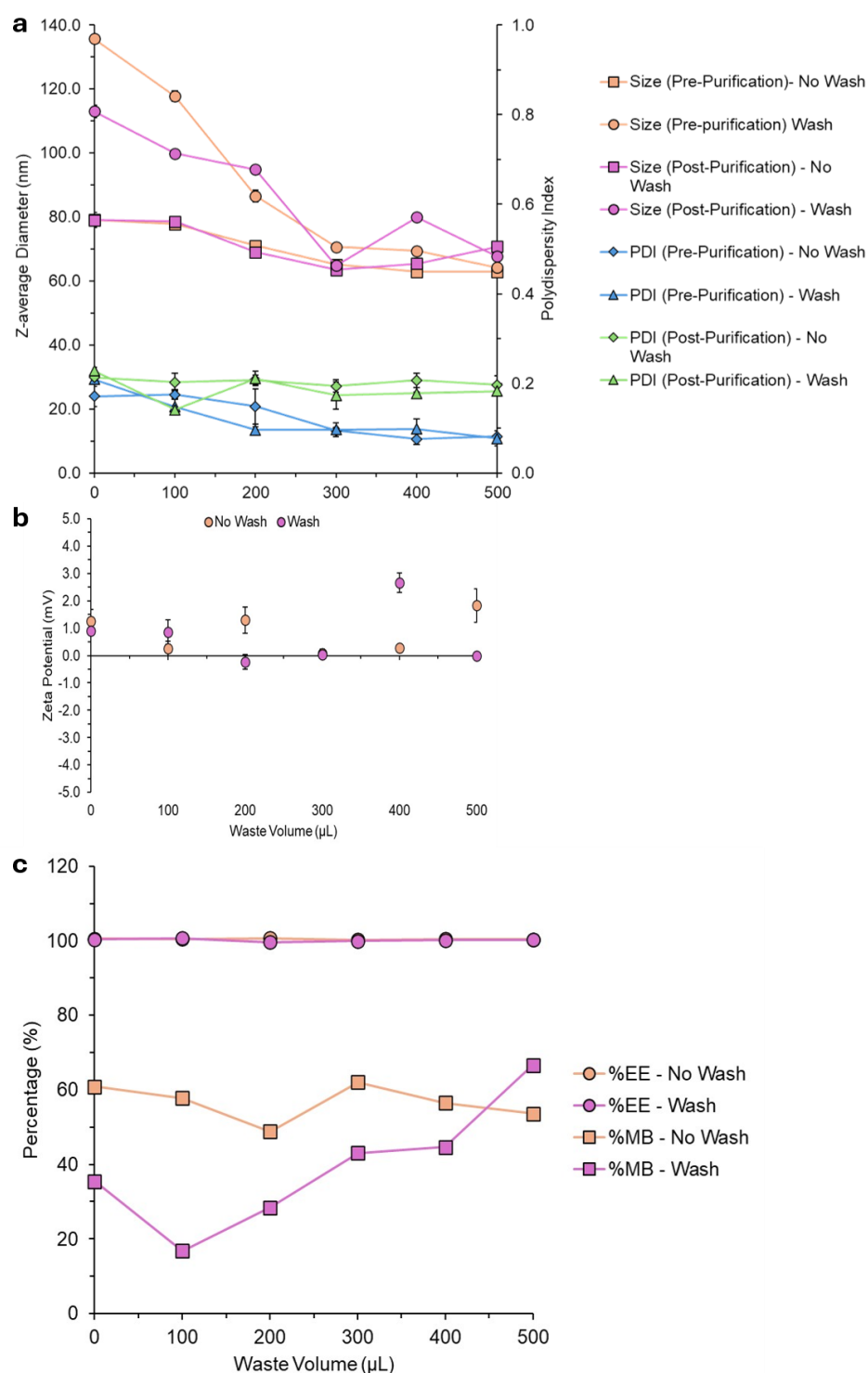


Figure 2-17 The Effect of Wastage on Continuous LNP Manufacture. LNPs consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ at a molar ratio of 10:38.5:50:1.5% encapsulating poly A to a final concentration of 0.025 mg/mL purified by dialysis. (a) represents the z-average diameter (nm) and polydispersity index, (b) represents the zeta potential (mV) and (c) represents poly A encapsulation efficiency (%) and mass balance (%). Results are representative of the mean $\pm$ standard deviation (SD) for technical replicates (n=1).

2.3.3 The Effect of Post-Processing Filtration

To conclude, once LNPs have undergone the appropriate CPP considerations and formulation testing, they must then undergo a range of post-processing steps to ensure that the samples are sterile and safe for clinical use. This often involves sterile syringe filtration of the LNPs to remove any residual particulates. While this step helps ensure LNPs meet regulatory compliance, it also introduces a possible drawback in terms of preserving LNP size and homogeneity, as well as encapsulation efficiency, which, in turn, will affect formulation efficacy.

To determine how filtration can affect the CQAs of LNPs, SM-102 LNPs were manufactured and purified before being subjected to syringe filtration through a 0.22 μm filter, which has either been left 'dry' or has been 'wet' prior to use with the buffer in which the sample is suspended. The resulting CQAs are seen in Table 2-3.

Table 2-3 highlights that when the filter is dry, the particle size suffers a non-significant increase ($p>0.05$) in size of around 60 nm with the PDI remaining steady, whereas when the filter is 'wet' and primed, the average particle size decreases by around 10 nm, with the PDI slightly increasing. The lack of statistical significance, even with larger changes, is largely due to the high variability observed in post-filtration samples, resulting in larger standard deviations. When considering zeta potentials, no negative effects were observed under either filtration condition tested (Table 2-3). For the primed filter condition, a larger decrease in encapsulation efficiency of ~25% was observed, whereas this was not evident for the dry filter. However, both tested conditions resulted in significantly ($p<0.01$) decreased poly A mass balance with decreases of around 30% (Table 2-3).

Overall, this data highlights how post-processing can introduce variability in LNPs and how formulations may require optimisation to enhance colloidal stability and maintain integrity.

Table 2-3 Critical Quality Attributes of LNPs manufacturing and subjected to Syringe Filtration. LNPs were manufactured on the NanoAssemblr® and consisted of DSPC:cholesterol:SM-102:DMG-PEG₂₀₀₀ encapsulating either poly A concentrated to a final concentration of 0.055 mg/mL. Results are representative of mean ± standard deviation (SD) (n=9).

	Pre-Filtration					Post-Filtration				
	Size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Mass Balance (%)	Size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Mass Balance (%)
Primed Filter	93.9 ± 11.6	0.128 ± 0.047	0.5 ± 1.8	101.7 ± 4.2	72.0 ± 10.4	85.6 ± 14.0	0.182 ± 0.213	2.1 ± 1.5	76.2 ± 43.5	45.8 ± 29.8
Dry Filter	74.1 ± 7.7	0.230 ± 0.048	4.0 ± 1.9	101.2 ± 2.9	61.7 ± 2.2	130.9 ± 191.4	0.226 ± 0.144	1.9 ± 3.3	101.3 ± 12.6	37.6 ± 20.5

2.4 Discussion

To summarise, the purpose of this chapter was to draw focus around several key CPPs in LNP formulation and manufacture with the aim to enhance knowledge of their effects and the result this could have on larger-scale manufacturing and to shape the studies undertaken during the subsequent work.

Initial investigations focused on phase preparation and how these formulation parameters affect the resulting LNPs. The lipid choice was shown to be a key driver of LNP CQAs, with ionizable lipids generally yielding larger LNPs with lower mass balance than a cationic lipid (Figure 2-2). Firstly, this research underpins data to follow in Chapter 4 where the results demonstrated that LNPs manufactured via pipette mixing decreased in size as specified lipids were employed (SM-102>DLin-MC3-DMA>DODAP>DOTAP), with DOTAP-LNPs exhibiting the highest payload encapsulation [165], overall agreeing with the conclusion seen here. Similarly, this is consistent with work by Binici et al., in which DOTAP particles were smaller, and no differences in payload mass balance were observed [77]. The difference in size can be largely accounted for by differences in charge between the lipids. Cationic lipids retain their positive charge regardless of environmental pH, which enhances electrostatic interactions with the payload and electrostatic repulsion between particles, leading to increased colloidal stability and smaller sizes compared to ionizable lipids, whose level of positive charge depends on environmental pH [171]. Despite slightly larger particles and lower mass balance, ionizable lipids continue to be the preferred choice for LNP formulation due to the toxicity associated with cationic lipids [77, 171, 172]. This highlights the need to further optimise formulation parameters to enhance these downfalls. Alteration of the lipid concentration has been shown to be a logical step in aiding this, with Terada et al. demonstrating through a DoE model that higher lipid concentrations and lower FFRs could result in increased siRNA-LNP size [173]. This disagrees with

the results observed in this chapter for lipid concentration x mixer type (Figure 2-3) and lipid concentration x TFR (Figure 2-4). However, Terada et al., considered a wider testing range of 10-30 mM, while only ~2-6 mg/mL were tested in this study. Indicating our results may be in a 'sweet spot' where concentration is negligible. On the contrary, Shkodra et al., were able to manufacture mRNA-LNPs up to an initial lipid concentration of 70 mg/mL with minimal effect on the CQAs [96].

Formulation parameter investigation also examines the effect of shifting the citrate buffer pH from 4 to 5 during LNP manufacture (Figure 2-5). Shifts to a near neutral pH are more beneficial for nucleic acid payloads due to their affinity to hydrolyse in low acidic environments [174]. However, acidic environments are essential for protonation of the ionizable lipids. The results were able to establish that this shift in pH was larger and unaffected (Figure 2-5). Previous work by Semple et al. established citrate pH as a decisive factor in DODAP particles, with encapsulation increasing significantly at lower pH [175]. This highlights a key benefit of ionizable lipids compared to cationic lipids, again enhancing their role in LNP formulation. In addition, within this chapter, the addition of Sucrose as a cryoprotectant was investigated to see if it affected LNP CQAs (Figure 2-6). It was established that the effect varied by formulation, with both formulation 1 and formulation 2 resulting in increased size. This agrees with what is normally seen in literature with Widayat et al., demonstrating that as sucrose concentration increases, so does mRNA-LNP size [176]. Widayat et al, stated that the effect sucrose has on LNP is not fully understood, but a plausible cause is interactions between the sucrose and the phospholipids, causing O-H bonds, which can lead to forms of lateral expansion [176, 177]. Additionally, the results demonstrated that the inclusion of sucrose could increase mRNA mass balance (Figure 2-6), which could be theorised to be due to increased viscosity during purification, making the samples more stable and able to withstand centrifugal forces.

Finally, fluorescent makers were investigated for their ability to monitor biodistribution and affect LNP CQAs. While the results showed that DiR and Cy5

were appropriate fluorescent labels for their intended purposes, both decreased *in vivo* expression (Figure 2-7) and increased size in the case of DiR (Figure 2-7). This effect was not seen for Ma et al., who manufactured SM-102 LNPs with both DiR and Cy5 as fluorescent labels [178]. Indicating that the effect of size could also be a function of the mixer used to manufacture, as the LNPs in Table 2 were manufactured with a cross-flow mixer, which generally gives sizes ~100 nm for mRNA-SM-102-LNPs [179], and the addition of the DiR could be causing some disruptions in the lipid membrane, leading to larger sizes. This lipid membrane disruption could lead to an altered LNP structure, increasing size and resulting in decreased *in vivo* expression. The trend of larger LNPs showing lower expression has been previously explored by McMillan et al., only as a function of FRR [105].

Advancing on from formulation CPPs, this chapter next investigated manufacturing CPPs. Arguably, the most vital of CPPs, first investigated was the effect of total flow rates (TFR) and flow rate ratios (FRRs). Extensive research into TFR and FRR as identified that increases in both generally lead to decreases in particle size and higher encapsulation efficiencies due to increases in solvent dilution and smaller mixing time scales, resulting in more efficient nanoparticle formation [180]. McMillan et al., generated a dataset which established that smaller ratios of 1:1 exhibited larger sizes, as well as decreased efficacy and altered structures compared to those of 3:1 LNPs [105]. This largely agrees with our research shown in Figure 2-15, indicating that the effect of FRR is larger, stable and not machine dependent. Roces et al., also demonstrated that increases in TFR and FRR could decrease particle size [106]. While this does not agree with our research, which shows that TFR does not largely affect LNPs during continuous increases (Figure 2-11), the design space is likely within a bench-scale TFR, where TFR changes are expected not to affect CQAs, but whether this would affect future large-scale manufacture would need consideration.

Microfluidic mixers x purification also does not appear to be a limiting factor of LNP CQAs as with the results demonstrating almost identical CQAs for SM-102 LNPs across two purification methods and three mixers (Figure 2-15). The key fact to identify is that spin column chromatography results in significant increases in size likely attributed to increased centrifugal forces and rapid changes in pH.

An under-reported factor that was identified as a key CPP was the wastage. First it was demonstrated that low-cost mixers, which generally have larger channels or longer tube lengths, are more affected by wastage and require ~500 μ L to maintain high payload mass balance (Figure 2-16). On the other hand, wastage in an impingement jet mixer becomes a significant CPP only when a wash step is included between samples (Figure 2-17), with ~400-500 μ L yielding the best payload mass balance under the tested conditions. Kis et al., compiled an extensive article looking at the resource and production costs associated with the global pandemic demand for LNPs [181]. They showed that variation in RNA amounts per dose was one of the larger annual production costs, and by optimising something as simple as head- and toe- wastage, this downfall could be avoided and enhance cost and time benefits.

Lastly, the results looked at how syringe filtration through a 0.22 μ m filter yielded non-significant but variable results when both a primed and a dry filter were used (Table 2-3). Dietrich et al., demonstrated non-significant changes to their LNP size after filtration [170]. While Messerian et al., completed a study on sterile filtration of mRNA-LNPs, showing no significant changes, but evidence of LNPs retained on the membrane was identified through SEM imaging [182].

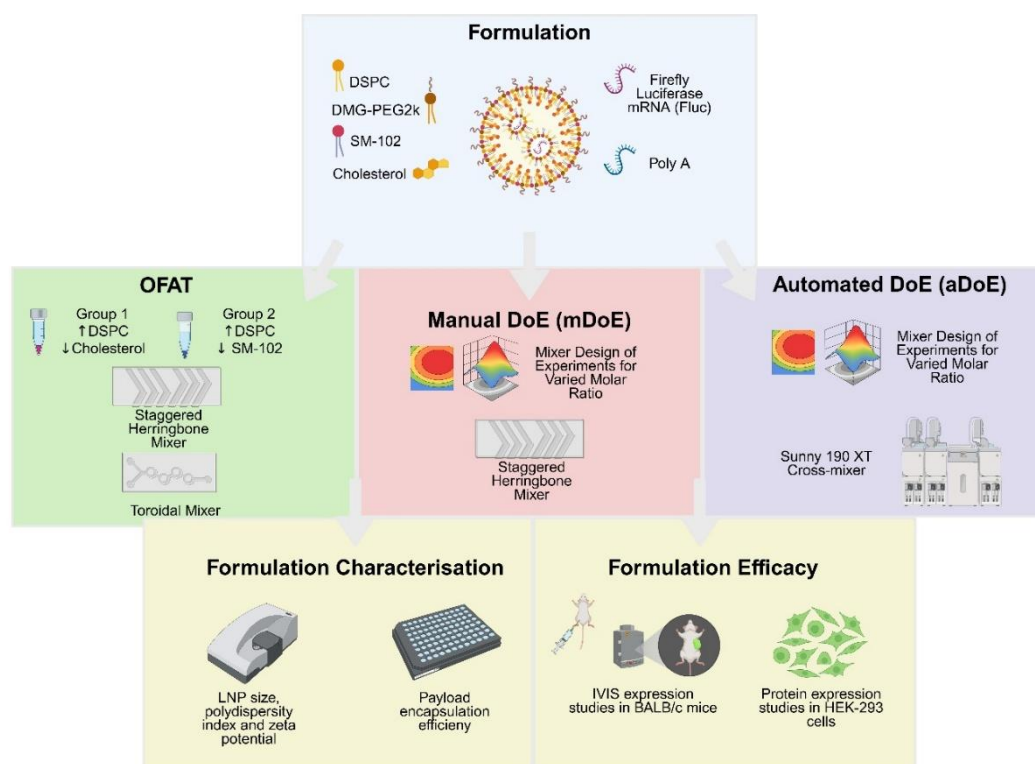
2.5 Conclusions

In conclusion, this chapter demonstrated that lipid nanoparticle CQAs are strongly governed by CPPs at the formulation, manufacturing, and post-processing stages. While it is often hard to establish one factor resulting in a key change, it is better to identify ‘sweet spots’ in which some CQAs may not matter

at bench-scale but could lead to lasting effects in GMP manufacture. Considered together, these results emphasise the need to define well-established process parameters to ensure LNPs are optimised for their intended use

Chapter 3: Revisiting the Lipid Ratio in Lipid Nanoparticles: A Comparison of OFAT and Design Experiments Approaches

Type of Publication	☒ Published article ☐ Reviewed for publication ☐ Submitted for publication ☐ Drafted for publication/application notes ☐ Not applicable
Citation Details (only applicable if published)	Forrester, J., Vaughan, E., Knappett, B.R. et al. Revisiting the lipid ratio in lipid nanoparticles: A comparison of OFAT and design of experiments approaches. Drug Deliv. and Transl. Res. (2026). https://doi.org/10.1007/s13346-026-02125-6
Chapter Inclusion Details	☒ This chapter is a word-for-word reproduction of the article reviewed for publication with minor wording changes. ☐ This chapter contains minor revision of the original publication ☐ This majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	CRediT Conceptualisation was carried out by J.F., E.V., B.R.K. and Y.P. Methodology was designed by J.F. and Y.P. Validation and formal analysis were performed by J.F. and Y.P. Investigation was conducted by J.F. and Y.P. Data curation was overseen by J.F. and Y.P. Preparation of the original draft was undertaken by J.F., E.V., B.R.K. and Y.P. Visualisation was handled by J.F. and Y.P. All authors have read and agreed to the published version of the manuscript.



3.1 Abstract

Lipid nanoparticles (LNPs) are currently the most clinically advanced lipid-based nucleic acid delivery system. However, efficient and reliable methods for formulation screening remain a key challenge. In this study, one-factor-at-a-time (OFAT) and Design of Experiments (DoE) strategies were applied to investigate how lipid molar ratio influences critical quality attributes (CQAs) and mRNA expression. In addition, manually executed and automated microfluidic manufacturing methods were compared to assess their impact on formulation–performance relationships. Across all strategies, multiple formulations beyond the standard molar ratio achieved comparable physicochemical properties, including particle size <120 nm, low polydispersity index (PDI <0.2), and high encapsulation efficiency (>75%). However, differences in CQAs and expression profiles were observed between manually and automatically manufactured DoE formulations, indicating that formulation performance is influenced by both composition and process conditions. DoE analysis revealed non-linear and process-dependent relationships between lipid composition and CQAs, with

limited predictive capability, particularly for biological outputs. Comparison of OFAT and DoE datasets demonstrated that while OFAT identifies directional trends within a constrained design space, DoE provides broader insight into interaction effects and formulation trade-offs. Overall, these findings demonstrate that the conventional lipid ratio represents a robust but non-optimal formulation and support a shift towards context-dependent, design-space-driven optimisation of LNP systems.

3.2 Introduction

Lipid nanoparticles (LNPs) are the most clinically advanced lipid-based nucleic acid therapeutics (NATs). They were developed to overcome key limitations of naked NATs, including rapid degradation and inefficient delivery of messenger RNA (mRNA) to target tissues [183]. Their clinical translation has been accelerated by incremental formulation development, culminating in the rapid deployment of LNP-based vaccines during the COVID-19 pandemic, supported by prior advances such as the development of Onpattro (Alnylam), the first siRNA-LNP formulation approved in 2018 [184].

The development of Onpattro provided a basis for many current LNP formulations, including Spikevax and mRESVIA. As a result, many contemporary mRNA-LNP systems are derived from a relatively narrow design space, with lipid compositions and molar ratios largely inherited from early siRNA formulations rather than systematically re-optimised for mRNA delivery. In general, for these LNPs, the current critical quality attribute (CQA) acceptance levels are typically defined as particle sizes <100 nm, polydispersity index <0.2, a near-neutral zeta potential, and high mRNA incorporation. These CQA thresholds, particularly LNP size, were primarily established to enable passage through liver fenestrae to facilitate hepatocyte targeting [169]. While appropriate for hepatic delivery, this design rationale may constrain formulation flexibility when alternative biodistribution or expression profiles are desired.

Two key drivers that affect the CQAs of LNPs are the manufacturing method and the formulation composition, including the lipid components and their relative ratios. Typically, LNPs consist of a 4-lipid mixture at a standard molar ratio of 10% phospholipid, 38.5% cholesterol, 50% ionisable lipid and 1.5% PEGylated lipid [22]. Figure 3-1 illustrates the lipids included in the approved formulations, with Onpattro, Spikevax, and mRESVIA closely reflecting this composition, and Comirnaty having a similar ratio but with decreased phospholipid and ionisable lipid content in exchange for increased cholesterol (9.4:42.7:46.3:1.5%, respectively) [88]. Despite these relatively small differences, the overall formulation paradigm remains largely conserved across approved products. However, this consistency also highlights the limited systematic exploration of alternative molar ratios in current LNP design.

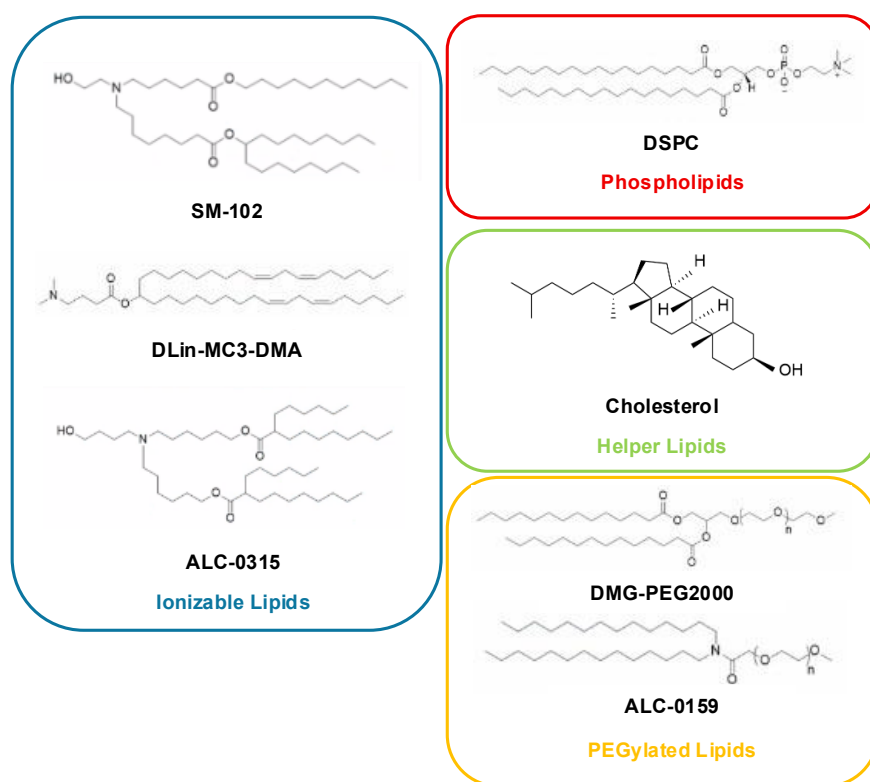


Figure 3-1 Chemical structures of lipids commonly used in clinically approved lipid nanoparticle (LNP) formulations. Ionisable lipids (SM-102, DLin-MC3-DMA and ALC-0315) are shown on the left, while helper lipids are shown on the right, including the phospholipid DSPC, cholesterol, and PEGylated lipids (DMG-PEG2000 and ALC-0159). Together, these four lipid classes form the basis of standard LNP formulations used for nucleic acid delivery. Structures were drawn using ChemDraw v23.1.2

While changes in lipid composition may appear minimal, they can drive key characteristics and functional effects that can influence LNP efficiency. Importantly, the structural and functional behaviour of LNPs is not solely dictated by a single lipid component in isolation, but rather by complex, non-linear interactions between lipids. Therefore, it is vital to understand the role of each lipid and their combinatorial effects to better define the flexibility of the molar ratio and to enhance the effectiveness of mRNA-LNPs.

Phospholipids, commonly DSPC, are included within LNPs to increase stability and payload encapsulation by enhancing the interaction of the phosphate backbone of the nucleic acid and the tertiary amine of the ionisable lipid [51] as well as aiding in destabilising the endosomal membrane, resulting in increased payload release and improved transfection efficiencies [22]. DSPC, along with cholesterol, constitutes nearly 50% of the formulation. Cholesterol exhibits a planar, rigid structure that allows it to ‘fill the gaps’ between phospholipid molecules in the LNP, thereby enhancing structural integrity [22] and increasing biocompatibility [52]. Increased cholesterol levels have also been shown to affect LNP structure and protein expression levels [58, 185]. The inclusion of DSPC at approximately 10% and cholesterol at around 30-40% has become a common feature of LNP formulations in both pre-clinical and clinical studies [76, 186].

Ionisable lipids are included to bind the nucleic acid payload and typically comprise most of the formulation (approximately 50%). The high molar percentage of ionisable lipids has been well documented in early siRNA-LNP research [187] and in more recent mRNA-LNP research [76]. Decreasing levels of ionisable lipids reduce binding capacity for the mRNA payload, which may affect LNP efficacy, as protein expression from mRNA depends on the amount of mRNA molecules that reach the translational machinery. Finally, LNPs also contain 1.5% PEGylated lipid, which provides stability and prevents aggregation [22, 52]. PEGylated lipids can also affect LNP particle size, with higher concentrations

yielding smaller particles [65]. It has also been observed that increased PEGylation can lead to increased mRNA expression, possibly due to longer circulation times and greater stability. However, the inclusion of PEGylated lipids also raises concerns about anti-PEG antibodies, which may cause allergic reactions and reduce overall transfection efficacy [63].

Despite the clinical success of current LNP formulations, there remains a need to revisit and challenge the accepted lipid molar ratio, particularly to improve extrahepatic targeting and enhance transfection efficacy. In parallel, there is a clear need for robust, scalable, and reproducible strategies for formulation screening at the bench scale. Traditionally, formulation optimisation is undertaken using one-factor-at-a-time (OFAT) approaches, in which one factor is varied while the others remain constant. OFAT approaches offer a practical and straightforward means of systematically analysing individual parameters; however, given the complexity of LNP systems and the extent of lipid-lipid interactions, this approach may overlook interaction effects and constrain exploration of the formulation design space [169]. Furthermore, the restricted experimental space inherent to OFAT can lead to repeated testing and fragmented interpretation, thereby limiting insight into formulation interdependencies.

These limitations emphasise the need for more robust and scalable formulation strategies, including Design of Experiments (DoE). DoE provides a systematic framework that maximises information gained from a reduced number of formulations [169] and enables the identification of interaction effects that are inaccessible using OFAT approaches. However, while DoE can provide broader insight into formulation behaviour, its ability to predict complex biological outcomes remains limited, as relationships identified using *in vitro* assays do not always translate to *in vivo* performance. This disconnect has been reported for nucleic acid nanoparticle systems, where DoE-optimised formulations showed poor correlation between *in vitro* transfection efficiency and *in vivo* expression,

highlighting the challenges of applying statistically optimised models to multifactorial biological systems [188].

Therefore, this study directly compares OFAT and DoE approaches within the same LNP system, focusing on how conclusions regarding lipid molar ratio, physicochemical properties, and *in vitro* and *in vivo* mRNA expression depend on both the formulation strategy and the manufacturing conditions employed. In addition, manually executed and automated microfluidic approaches were evaluated to assess the influence of process conditions on formulation–performance relationships. By revisiting the established lipid ratio paradigm, this work provides comparative insights into formulation screening strategies, while contributing to the existing knowledge on how molar ratios affect LNP physicochemical properties and *in vitro/in vivo* protein expression.

3.3 Materials and Methods

3.3.1 Materials

The phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and the PEGylated lipid 1,2-dimyristoyl-sn-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were acquired from Avanti Polar Lipids (Alabaster, AL, USA), while the ionisable lipid Heptadecan-9-yl 8-((2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino) octanoate (SM-102) was purchased from Broadpharm (San Diego, CA, USA). Heat-inactivated fetal bovine serum (FBS), cholesterol, polyadenylic acid (Poly A), sodium citrate dihydrate, citric acid and HPLC-grade methanol were obtained from Merck Life Science UK Ltd. (Gillingham, UK). Ethanol was purchased from Supelco (Bellefonte, PA, USA). The nucleic acid EZ Cap™ Firefly Luciferase mRNA (5-moUTP) was purchased from APEX BIO (Houston, TX, USA). The Quanti-iT Ribogreen RNA Assay kit, triethylamine acetate buffer, and Tris-HCl buffer were purchased from Thermo Fisher Scientific (Invitrogen, Waltham, MA, USA). Cell culture reagents, including Minimal Essential Medium (MEM), sodium pyruvate, penicillin/streptomycin, and TrypLE,

were purchased from Gibco Technologies, while HEK-293 cells were obtained from the American Type Culture Collection (ATCC) (CRL-1573). Finally, the Rabbit Reticulocyte Lysate Kit and ONE-Glo™ Luciferase Assay System were purchased from Promega Ltd. (Chilworth, UK). Deionised and ultrapure water were supplied through an in-house system.

3.3.2 Microfluidic Manufacture of Lipid Nanoparticles (LNPs)

3.3.2.1 One-Factor at a Time (OFAT)

OFAT LNPs were manufactured using the Ignite™ or NanoAssemblr Benchtop® (Precision Nanosystems Inc, Vancouver, Canada) using a flow rate ratio (FRR) of 3:1 (aqueous:lipid) and a total flow rate (TFR) of 15 mL/min. The aqueous phase consisted of either Firefly Luciferase mRNA (Fluc) or Poly A in 50 mM citrate buffer at pH 5. The lipid phase consisted of DSPC, cholesterol, SM-102, and DMG-PEG2k at varying molar ratios (Table 3-1) in ethanol. LNPs were produced at a w/w ratio of 22:1 (lipid:mRNA), which corresponded to a final concentration of 0.055 mg/mL payload and 1.25 mg/mL lipid. For *in vivo* studies, formulations were adjusted to account for encapsulation efficiency, and all LNPs were normalised to a final mRNA concentration of 0.1 mg/mL prior to administration.

Table 3-1 Lipid Compositions of LNP Formulations manufactured during OFAT Testing. Each formulation is assigned an LNP number for easy identification, and individual lipid proportions are detailed in the remaining columns.

LNP Number	DSPC (%)	Cholesterol (%)	SM-102 (%)	DMG-PEG2k (%)
LNP1	10	38.5	50	1.5
LNP2	20	28.5		
LNP3	30	18.5		
LNP4	40	8.5		
LNP5	48.5	-		
LNP6	-	48.5		
LNP7	20	38.5	40	
LNP8	30		30	
LNP9	40		20	
LNP10	60		-	

3.3.2.2 Manually Executed Microfluidic Mixing Design of Experiments (mDoE)

mDoE LNPs were manufactured using the NanoAssemblr Benchtop® (Precision Nanosystems Inc, Vancouver, Canada) at a FRR of 3:1 (aqueous:lipid) and TFR of 15 mL/min. The aqueous phase contained Firefly Luciferase mRNA (Fluc) in 50 mM, pH 5 citrate buffer, while the lipid phase consisted of DSPC, cholesterol, SM-102, and DMG-PEG2k at varying molar ratios (Table 3-2). LNPs were produced at a constant w/w ratio of 22:1 (lipid:mRNA), which corresponded to a final concentration of 0.025 mg/mL mRNA and 0.55 mg/mL lipid.

3.3.2.3 Automated Design of Experiments (aDoE)

aDoE LNPs were manufactured using the Sunscreen™ microfluidic mixer with a Sunny 190XT T-mixing channel (Unchained Labs, California, USA) at a FRR of 3:1 (aqueous:lipid) and a TFR of 12 mL/min. This microfluidic platform differs from the NanoAssemblr Benchtop in channel geometry, flow dynamics, and post-processing steps, which were maintained as per manufacturer protocols. The aqueous phase consisted of Firefly Luciferase mRNA in 50 mM pH 5 citrate buffer, while the lipid phase again consisted of DSPC, cholesterol, SM-102 and DMG-PEG2k at varied molar ratios (Table 3-2). LNPs were manufactured at a w/w ratio of 22:1 (lipid:mRNA) corresponding to 0.04 mg/mL mRNA and 0.9 mg/mL lipid, which were concentrated 4x using spin columns to achieve 0.16 mg/mL mRNA and 3.6 mg/mL lipid.

Table 3-2 Lipid Compositions for LNPs Manufactured During DoE Testing. LNPs can be identified using the LNP number followed by 'm' to indicate they were manufactured manually or 'a' to indicate automated manufacture.

LNP Number	DSPC (%)	Cholesterol (%)	SM-102 (%)	DMG-PEG2k (%)
LNP1m/a	24.38	40.53	34.17	0.92
LNP2m/a	14.13	51.53	33.42	0.92
LNP3m/a	24.13	40.53	33.42	1.92
LNP4m/a	9.50	40.00	50.00	0.50
LNP5m/a	24.39	40.53	33.42	1.67
LNP6m/a	9.50	58.00	30.00	2.50
LNP7m/a	30.00	39.50	30.00	0.50
LNP8m/a	14.13	50.53	33.42	1.92
LNP9m/a	9.50	38.00	50.00	2.50
LNP10m/a	15.13	40.53	43.42	0.92
LNP11m/a	9.50	60.00	30.00	0.50

LNP12m/a	30.00	38.00	30.00	2.00
LNP13m/a	29.50	38.00	30.00	2.50
LNP14m/a	14.13	41.53	43.42	0.92
LNP15m/a	11.50	38.00	50.00	0.50
LNP16m/a	18.78	43.06	36.83	1.33
LNP17m/a	14.13	40.53	43.42	1.92
LNP18m/a	30.00	38.00	31.50	0.50
LNP19m/a	24.38	41.28	33.42	0.92

3.3.3 Buffer Exchange and Purification

LNPs purified using centrifugal filtration with Amicon Ultra-15 spin columns were diluted 1:40 (v/v) with 10 mM Tris buffer at pH 7.5 and then subjected to centrifugal filtration at $2000 \times g$ at 4 °C until the desired volume was recovered. Some LNPs were further concentrated (2x or 4x) for *in vivo* and aDoE studies. LNPs purified using dialysis were placed in a dialysis membrane with a 14 kDa molecular weight cut off and dialysed against 10 mM pH 7.5 Tris buffer under magnetic stirring for 4 hours at room temperature (poly A-LNPs) or 4 °C (mRNA-LNPs).

3.3.4 LNP Physicochemical Characterisation

LNPs were characterised using both the Malvern Zetasizer Ultra and the Zetasizer ZS, with a 173° backscattering angle, with dynamic light scattering (DLS) used to measure size/polydispersity and electrophoretic light scattering (ELS) to measure zeta potential. LNP size and polydispersity were measured in a ZEN0040 micro-cuvette at a lipid concentration of 0.1 mg/mL, using citrate buffer (post-microfluidic) and Tris buffer (post-purification). The viscosity and refractive index of both diluents were 1.28 cP and 1.47, and 1.0037 cP and 1.337, respectively. Zeta potential measurements were carried out using a DTS1017 folded capillary cell at a lipid concentration of 0.1 mg/mL in water with a viscosity and refractive index of 0.8872 cP and 1.33.

3.3.5 Quantification of Nucleic Acid Encapsulation

Nucleic acid encapsulation was quantified using the Quanti iT™ Ribogreen™ RNA Assay Kit (Invitrogen™). Standard curves were prepared over appropriate concentration ranges for each nucleic acid payload. Briefly, samples were diluted to a payload concentration of 3 µg/mL, mixed with either 50 µL of 2% Triton-TE or 1X TE buffer, then incubated for 15 minutes at 37 °C. Fluorescence was measured using the Glomax (Promega) at excitation 475 nm and emission 500-550 nm.

3.3.6 *In vitro* Luciferase Assay

HEK293 cells were seeded in a white, clear-bottom 96-well plate, at a density of 1×10^5 cells/well in minimum essential media (MEM) (10% Foetal Bovine Serum, 1% sodium pyruvate, 1% penicillin streptomycin) and incubated for 48 h at 37 °C, 5% CO₂. Following incubation, LNPs were serially diluted in MEM (mRNA concentrations of 2 µg/mL, 1 µg/mL, 0.5 µg/mL, and 0.25 µg/mL), and 100 µL of each concentration was added to the wells in triplicate, which were then incubated for a further 24 h under the same conditions. After incubation, 100 µL of ONE-glo™ luciferase substrate was added to all wells; a non-treated control was included, and luminescence was measured using a GloMax (Promega).

3.3.7 Cell-Free Protein Expression

Cell-free protein expression (CFPE) was analysed using the Rabbit Reticulocyte Lysate System (Promega). mRNA was extracted from LNPs using a two-step ethanol precipitation protocol. LNP samples were diluted to 10 µg/mL mRNA in Tris buffer, combined with ethanol and sodium acetate, and centrifuged at 14,000 rpm for 20 min. Ethanol precipitation was carried out twice then the resulting pellet was washed and resuspended in RNase-free water. Extracted mRNA was diluted to 2 µg/mL and added to reaction mixtures as detailed in Table 3-3. All samples were normalised to equivalent mRNA input prior to analysis. Reaction mixtures were incubated at 30 °C for 1.5 h, then terminated by placing the samples on ice. Protein expression was quantified by mixing reaction mixtures

1:1 (v/v) with ONE-Glo™ luciferase substrate and measuring luminescence using a GloMax (Promega).

Table 3-3 CFPE Reaction Mixtures. This table details the required component quantities for the cell-free protein expression reaction used to monitor protein expression in extracted mRNA samples.

Component	Standard Reaction	Positive Control	Negative Control
Rabbit Reticulocyte Lysate	35 µL	35 µL	35 µL
Amino Acid Mixture Minus Leu.	0.5 µL	0.5 µL	0.5 µL
Amino Acid Mixture Minus Met.	0.5 µL	0.5 µL	0.5 µL
RNasin® Ribonuclease Inhibitor	1 µL	1 µL	1 µL
Firefly Luciferase mRNA	-	2 µL	-
Extracted Firefly Luciferase mRNA	2 µL	-	-
RNase Free Water	11 µL	11 µL	13 µL

3.3.8 *In vivo* Studies

For the OFAT LNPs, to evaluate the *in vivo* biodistribution and expression efficiency of the mRNA-LNPs, 12–17-week-old male BALB/c mice were injected bilaterally with 50 µL/leg of mRNA-LNPs intramuscularly equating to 10 µg of firefly luciferase mRNA per mouse. The mice were then subcutaneously injected with VivoGlo D-Luciferin (Promega, UK) at a concentration of 30 mg/mL (5 µL/g), and bioluminescent signals were detected using the IVIS Spectrum (PerkinElmer). At the 6 h time point after intramuscular injection, mice were once again imaged under the bioluminescence filter following subcutaneous injection with VivoGlo D-luciferin substrate, and bioluminescence signals were again measured under the bioluminescence filter.

For the aDoE LNPs, 16-17-week-old male BALB/c mice were used to evaluate *in vivo* biodistribution. Each mouse received the LNPs via an intramuscular injection in the leg. Mice received 50 µL/leg of LNPs, with each leg receiving a distinct LNP formulation. Mice were then injected subcutaneously with D-luciferin and imaged 10 min later. Imaging was repeated at 6 h post-injection. A control mouse that did not receive LNPs was included for baseline comparison.

Bioluminescence signals were quantified using Living Image Software (v4.0). Differences between OFAT and aDoE studies reflect variations in formulation concentration and experimental design required for each dataset.

3.3.9 High Performance Liquid Chromatography - Evaporated Light Scattering Detector (HPLC-ELSD)

Lipid quantification was performed using a previously reported HPLC-ELSD method [165]. LNP samples were analysed using the Shimadzu HPLC-ELSD III system. Samples were injected at 10 μ L with a flow rate of 0.5 mL/min. Lipids were separated using a Waters XBridge[®] C18 column (3 mm $\times$ 150 mm, 130 Å pore size, 3.5 μ m particle size) with a detector temperature of 50 °C and column oven at 45 °C. Mobile phase A consisted of 0.01 M triethylamine acetate in water, and mobile phase B consisted of 0.01 M triethylamine acetate in ethanol. A binary gradient was applied, increasing mobile phase B from 70% to 100% over 0–7 min, then returning to the initial conditions. Samples were diluted 1:5 (v/v) in mobile phase B prior to analysis. Calibration curves were prepared for each lipid. This method was used to assess lipid recovery and to confirm the formulation composition of manually prepared LNPs.

3.3.10 Design of Experiments (DoEs)

Minitab was used to construct a mixture DoE, ensuring that the total lipid composition was constrained to 100% and that the run order was randomised. The four input parameters were DSPC (9.5–30%), cholesterol (38–60%), SM-102 (30–50%), and DMG-PEG2000 (0.5–2.5%). The design generated 19 formulations, and an additional formulation representing the standard molar ratio was included as a test standard (aDoE only). Response variables included particle size, PDI, zeta potential, encapsulation and *in vitro/in vivo* protein expression.

3.3.11 Statistical Analysis

Statistical significance was assessed using Minitab (version 19.2). Parametric data were analysed using ANOVA followed by Tukey post-hoc test, while non-parametric data were analysed using a Kruskal-Wallis test followed by Dunn's post-hoc test. All results are presented as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM), as specified in figure legends, with corresponding sample sizes (n) indicated. For the DoE models, predicted accuracy was defined as agreement between predicted and experimentally measured values within 80-120%. This threshold was selected as a pragmatic range to account for experimental and biological variability while enabling meaningful assessment of model performance.

3.4 Results

3.4.1 The Impact of Molar Ratio on LNP Physicochemical Properties

Due to the limited systematic exploration of mRNA-LNP formulations with varied lipid molar ratios, the effects of altering the standard molar ratio on critical quality attributes (CQAs) and both *in vitro* and *in vivo* performance remain incompletely characterised. To address this gap, an OFAT approach was applied, varying DSPC content at the expense of either cholesterol (group one; LNP1–6) or SM-102 (group two; LNP7–10), relative to a standard formulation (LNP1; 10:38.5:50:1.5%) (Figure 3-2).

Figure 3-2 demonstrates that, across both groups, most formulations produced Poly A-loaded LNPs in the 80–120 nm size range with low polydispersity (PDI < 0.2), indicating consistent particle formation across the tested compositional space (Figure 3-2a,b). For group one (LNP1 – 6), increasing DSPC at the expense of cholesterol resulted in relatively minor changes in particle size and PDI. The largest LNP size was observed for LNP6, in which DSPC was removed and cholesterol content increased; however, this size increase was not significantly

different from that of the standard formulation (LNP1) (Figure 3-2a). In contrast, removal of cholesterol (LNP5) resulted in a significant increase in PDI ($p < 0.05$), indicating reduced uniformity of the particle population (Figure 3-2a). For group two (LNP7–10), increasing DSPC at the expense of SM-102 also resulted in relatively consistent particle sizes for LNP7, LNP8 and LNP9, although greater variability in the size range is noted for LNP8. Furthermore, LNP10, which contained no ionisable lipid, exhibited a marked reduction in particle size (<70 nm) compared to other formulations ($p < 0.05$) (Figure 3-2b).

Zeta potential measurements showed that group one formulations (LNP1–6) exhibited similar positive charge with no significant differences between formulations (Figure 3-2c). In group two, LNP7–9 also showed comparable zeta potential, whereas LNP10 (which contains no ionisable lipid) exhibited a shift towards negative zeta potential (Figure 3-2d).

Encapsulation efficiency remained high ($>90\%$) across LNP1–9 (Figure 3-2e,f), indicating robust nucleic acid incorporation across most formulations. In contrast, LNP10 showed negligible encapsulation, consistent with the absence of ionisable lipid required for nucleic acid complexation.

Based on these results, LNP1, 3, 5, 6, 8, and 9 were selected for further evaluation. Selection was based on a combination of acceptable CQAs (particle size <120 nm, low PDI, and high encapsulation efficiency), alongside inclusion of formulations representing key compositional variations, including removal of individual lipid components and reduced ionisable lipid content, to enable targeted comparison of formulation–performance relationships in subsequent *in vitro*, *ex vivo*, and *in vivo* studies.

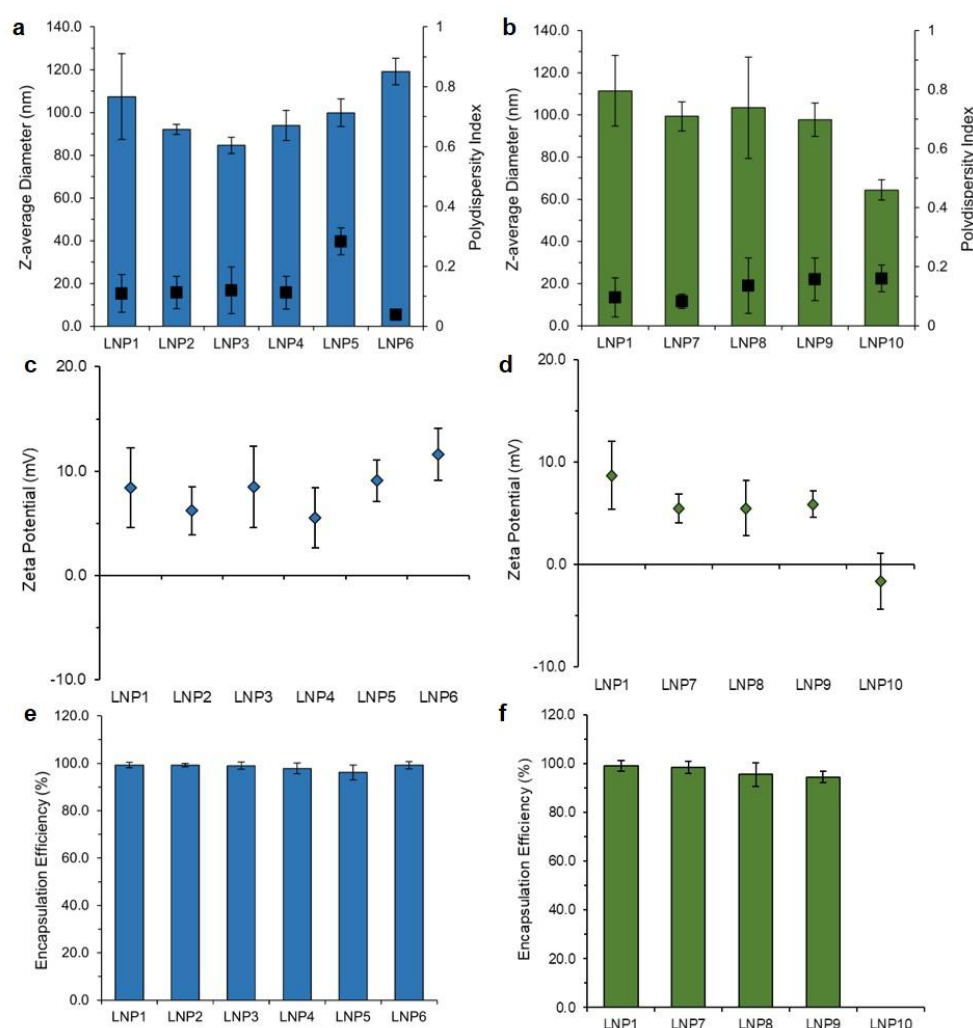


Figure 3-2 Physicochemical Characterisation and mRNA encapsulation of Group One and Group Two LNPs. LNPs were composed of DSPC:cholesterol:SM-102:DMG-PEG2000, including a standard formulation (10:38.5:50:1.5%) and variants in which DSPC content was increased from 20% to 60% at the expense of either cholesterol (Group One; LNP2–6) or SM-102 (Group Two; LNP7–10), while DMG-PEG2000 was maintained at 1.5%. Poly A was encapsulated in all formulations. (A, B) Particle size (bars) and polydispersity index (points) for group one (A) and group two (B). (C, D) Zeta potential for group one (C) and group two (D). (E, F) poly A encapsulation efficiency for group one (E) and group two (F). Data are presented as mean $\pm$ standard deviation ($n = 3-4$).

3.4.2 The *In vitro In vivo* Efficacy of Down-Selected LNPs

Following down-selection, LNP formulations encapsulating Firefly luciferase mRNA (Fluc) were manufactured and evaluated for physicochemical properties, *in vitro* expression, cell-free protein production, and *in vivo* performance (Figure

3-3). The selected formulations represent targeted variations in lipid composition relative to the standard molar ratio (LNP1), including reduced cholesterol (LNP3), complete cholesterol removal (LNP5), removal of DSPC (LNP6), and reduced ionisable lipid content (LNP8, 30%; LNP9, 20%) (Table 3-1).

Physicochemical characterisation of Fluc-loaded LNPs (Figure 3-3a–c) showed that most formulations maintained comparable particle size (80 – 120 nm) and PDI (< 0.2) to Poly A-loaded LNPs (Figure 3-2a), indicating that incorporation of mRNA did not substantially alter particle formation under standard conditions. An exception was observed for LNP5 (no cholesterol), which exhibited a notable increase in particle size and PDI variability when formulated with mRNA compared to Poly A (Figure 3-3a vs Figure 3-2a). Encapsulation efficiencies remained high (>94%) across LNP1–8 (Figure 3-3b), consistent with Poly A-loaded formulations (Figure 3-2), where both LNP8 (30% ionisable lipid) and LNP9 (20%) showed high encapsulation. However, for mRNA, encapsulation efficiency was maintained for LNP8 but was markedly reduced for LNP9 (~41%), suggesting that the impact of reduced ionisable lipid content may be more pronounced when encapsulating mRNA compared to Poly A. Zeta potential measurements (Figure 3-3c) showed no significant differences between formulations, with all LNPs exhibiting similar surface charge profiles, consistent with Poly A-loaded formulations (Figure 3-2c–d). Overall, incorporation of mRNA revealed greater formulation-dependent differences than Poly A, suggesting that the nature of the encapsulated nucleic acid can influence the extent to which compositional variations affect LNP properties.

In vitro luciferase expression (Figure 3-3d) showed that LNP1 (standard molar ratio) achieved high expression across the tested concentration range. Reducing cholesterol content (LNP3) or removing it entirely (LNP5) resulted in lower expression compared to LNP1. Removal of DSPC (LNP6) resulted in expression levels comparable to those of the standard LNP1 formulation. A reduction in ionisable lipid content led to decreased expression, as observed in LNP8 (30%)

and LNP9 (20%). No significant differences in cell viability were observed across formulations, indicating that changes in lipid composition did not adversely affect cellular viability under the conditions tested (data not shown). Based on reduced encapsulation efficiency and lower *in vitro* expression, LNP9 was excluded from further evaluation.

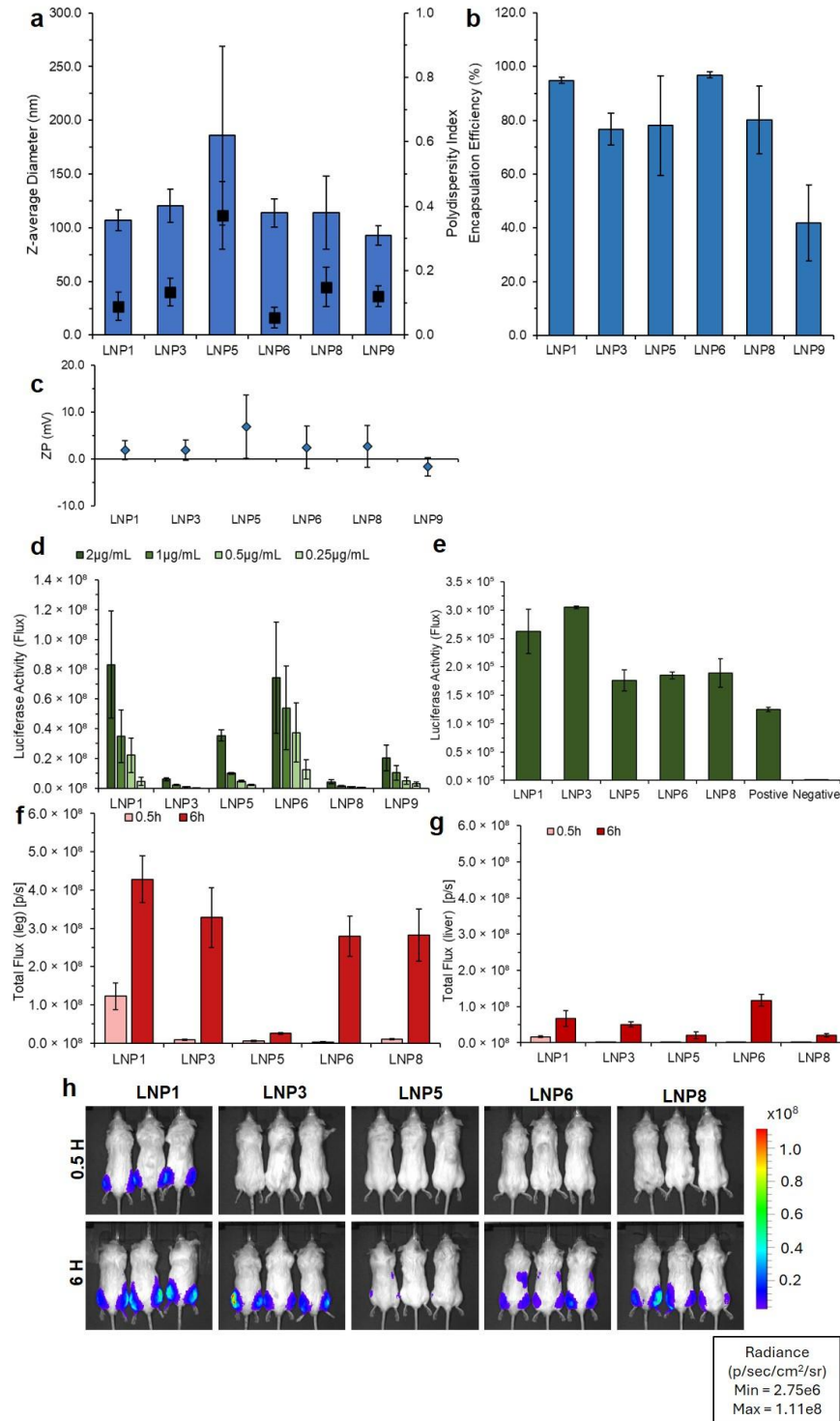


Figure 3-3 Physicochemical Characterisation and Biological Performance of Down-Selected LNP Formulations. LNPs composed of DSPC:cholesterol:SM-102:DMG-PEG2000 at varied molar ratios (LNP1, 3, 5, 6, 8 and 9; see Table 3-1) were evaluated. (a–c) Z-average diameter, polydispersity index (PDI), mRNA encapsulation efficiency, and zeta potential. (d) In vitro protein expression measured as luciferase activity. (e) Ex vivo protein expression measured using a cell-free protein expression (CFPE) system. (f, g) Quantified bioluminescence signals at 0.5 h and 6 h post-intramuscular injection at the injection site (f) and liver (g). The colour scale represents luminescence intensity from 0.2×10^8 (blue; minimum radiance 2.75×10^6 p/s/cm²/sr) to 1.0×10^8 (red; maximum radiance 1.11×10^8 p/s/cm²/sr). Representative IVIS images are shown in (h). Physicochemical data are presented as mean $\pm$ SD ($n = 3$ –6); in vitro and ex vivo data as mean $\pm$ SEM ($n = 2$ –4); in vivo data as mean $\pm$ SEM from two independent batches ($n = 12$ injection site; $n = 6$ liver).

Cell-free protein expression was assessed using a cell-free protein expression (CFPE) system (Figure 3-3e) to evaluate the translational capacity of mRNA recovered from each LNP formulation. All samples supported protein expression, indicating that mRNA remained functional following formulation and extraction. LNP1 and LNP3 exhibited higher mean expression than the positive control (naked mRNA), while LNP5, LNP6 and LNP8 showed similar expression to the positive control. Variability in CFPE output likely reflects differences in encapsulation efficiency and mRNA recovery during extraction, rather than intrinsic differences in translational capacity.

In vivo bioluminescence imaging (Figure 3-3f–h) revealed formulation-dependent differences in expression profiles. At 0.5 h post-injection, LNP1 exhibited significantly higher expression at the injection site compared to LNP3, LNP5 and LNP6 ($p < 0.05$), indicating more rapid early expression from the standard formulation. By 6 h, expression levels for LNP1, LNP3, LNP6, and LNP8 were comparable, suggesting convergence of expression over time, while LNP5 (no cholesterol) remained significantly lower than all formulations ($p < 0.01$).

Analysis of systemic (liver-associated) expression showed low overall levels across formulations. At 0.5 h, LNP1 exhibited significantly higher liver expression compared to all other formulations ($p < 0.01$). By 6 h, these differences were reduced, with LNP1, LNP3, and LNP6 exhibiting comparable expression levels, while LNP5 and LNP8 remained significantly lower than LNP6 (Figure 3-3g; $p < 0.01$). Representative *in vivo* images (Figure 3-3h) illustrate the spatial and temporal distribution of expression across formulations.

3.4.3 Loss of Functional Activity is not caused by a Loss of Component Integrity

Lipid recovery and composition were quantified by HPLC-ELSD to determine whether the reduced functional activity and lack of *in vitro*–*in vivo* correlation (IVIVC) observed for selected formulations were associated with loss of

component integrity. Figure 3-4 shows the recovered mass ratios of the LNPs, while Table 3-4 presents the recovery percentages for individual lipids in the formulation.

Total lipid recovery exceeded 80% for all formulations, ranging from $81 \pm 4\%$ (LNP6) to $106 \pm 31\%$ (LNP8), with no formulation-dependent trend (Table 3-4). Individual lipid recoveries were similarly consistent across formulations, with SM-102 and DMG-PEG2k recovered within $\sim 74\text{--}103\%$ and $\sim 82\text{--}100\%$, respectively. Cholesterol recovery ranged from $75 \pm 14\%$ (LNP9) to $92 \pm 3\%$ (LNP1). Greater variability was observed for DSPC, particularly in LNP8 ($130 \pm 76\%$), although this was associated with high standard deviation.

Recovered molar ratios (Figure 3-4) closely matched expected compositions for LNP1, LNP3, LNP5, and LNP6, demonstrating minimal deviation from target formulations. While increased DSPC proportions were observed in LNP8 and LNP9 relative to expected values, these shifts did not substantially disrupt the overall lipid balance.

No consistent loss of any individual lipid component was observed across formulations, and total lipid recovery remained high. As lipid recovery was confirmed to be consistent in these initial studies, and no systematic differences in biological performance were attributable to compositional loss, lipid quantification was not repeated for subsequent DoE formulations.

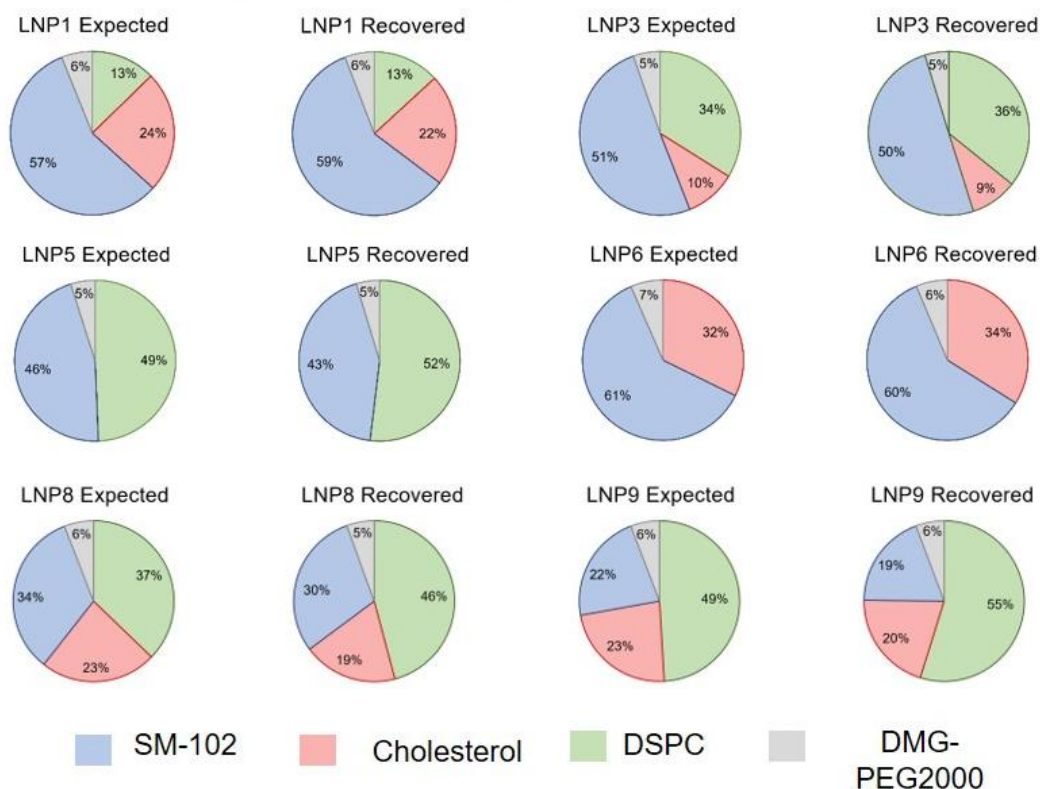


Figure 3-4 Lipid Quantification of Down-Selected LNPs. LNP formulations consist of polyA encapsulated within LNPs containing DSPC:cholesterol:SM-102:DMG-PEG2000 at varied molar ratios: LNP1 (10:38.5:50:1.5%), LNP3 (30:18.5:50:1.5%), LNP5 (48.5:0:50:1.5%), LNP6 (0:48.5:50:1.5%), LNP8 (30:38.5:30:1.5%) and LNP9 (40:38.5:20:1.5%). Results show the expected and recovered mass ratios of lipids in the LNP formulations, as determined by HPLC-ELSD. Results are presented as mean $\pm$ standard deviation (SD) ($n = 3$).

Table 3-4 Lipid Recovery of individual lipids within the formulation, detected and quantified using an HPLC-ELSD protocol. Results representative of the mean $\pm$ standard deviation (SD) ($n=3$).

	Recovered (%) $\pm$ SD				
	DSPC	Cholesterol	SM-102	DMG-PEG2k	Total
LNP1	104 $\pm$ 6	92 $\pm$ 3	103 $\pm$ 9	94 $\pm$ 5	100 $\pm$ 5
LNP3	96 $\pm$ 25	84 $\pm$ 13	89 $\pm$ 7	84 $\pm$ 21	90 $\pm$ 12
LNP5	96 $\pm$ 23	-	87 $\pm$ 17	87 $\pm$ 20	91 $\pm$ 18
LNP6	-	84 $\pm$ 7	79 $\pm$ 4	83 $\pm$ 7	81 $\pm$ 4
LNP8	130 $\pm$ 76	88 $\pm$ 13	95 $\pm$ 4	100 $\pm$ 18	106 $\pm$ 31
LNP9	95 $\pm$ 39	75 $\pm$ 14	74 $\pm$ 4	82 $\pm$ 15	85 $\pm$ 24

3.4.4 Rational for Transition from OFAT to DoE

While the preceding OFAT studies provided a useful baseline for understanding how variation in lipid molar ratios influences CQAs and formulation efficacy, this approach is inherently limited in capturing interaction effects between components. Such interactions are particularly important in multicomponent systems such as LNPs. Where lipid-lipid interactions can influence physicochemical properties and biological performance. In addition, the PEGylated lipid content was fixed at 1.5% throughout the OFAT studies, restricting insight into its potential contribution to formulation behaviour and representing a notable gap in the design space explored.

To address these limitations, a DoE approach was implemented to enable a more comprehensive and statistically robust evaluation of formulation variables. A mixture DoE was selected, as this allows lipid components to be varied simultaneously while constraining the total molar composition to 100%, thereby reflecting the inherent interdependence of formulation components. This approach was specifically chosen to quantify interaction effects and to facilitate the development of predictive models for formulation optimisation.

The mixture DoE generated 19 formulations, which were manufactured using either a manually operated microfluidic mixer or an automated microfluidic mixer. The resulting formulations were denoted as mLNPs and aLNPs, respectively. Both formulation sets were characterised with respect to the key CQAs identified earlier: particle size, PDI, zeta potential, and mRNA encapsulation efficiency, as well as *in vitro* transfection efficacy. In addition, the aLNPs formulations were progressed to *in vivo* evaluation to assess their translational relevance. Experimental data were subsequently analysed using Minitab to generate predictive models and assess the significance of individual factors and their interactions. Model validation was performed to evaluate the reliability of predictions within the explored design space. Therefore, this

approach enables a direct comparison with OFAT-derived insights. While providing a framework to evaluate whether increased experimental complexity translates into improved predictive power. CQA values for the manually manufactured DoE formulations (mLNPs) are summarised in Figure 3-10. Most formulations exhibited particle sizes below 200 nm, PDI values below 0.2, near-neutral zeta potentials, and encapsulation efficiencies greater than 75%, indicating that most formulations remained within acceptable physicochemical ranges. These data support the trends observed in the Cox response trace plots.

3.4.5 Composition-Response Relationships in Manual DoE LNPs (mLNPs)

The influence of lipid composition on LNP properties was evaluated using Cox response trace plots (Figure 3-5), enabling assessment of component sensitivity relative to the reference formulation. Across all responses, these plots illustrate how variation in the relative proportions of lipid components influences physicochemical properties and protein expression within the design space explored.

Particle size showed clear dependence on lipid composition (Figure 3-5a). Shifts towards higher DSPC and SM-102 proportions were associated with larger particle sizes, whereas shifts towards higher cholesterol or DMG-PEG2k resulted in smaller particle sizes. In contrast to the OFAT study, where variation in DSPC and cholesterol resulted in relatively minor changes in particle size, the DoE model indicates greater sensitivity to compositional variation across the broader design space, including effects associated with DMG-PEG2k that were not explored in the OFAT approach.

PDI exhibited minimal variation across the design space (Figure 3-5b). Among the lipid components, variation of DMG-PEG2k was associated with the most notable changes in PDI, while DSPC, cholesterol, and SM-102 showed more limited influence.

Zeta potential remained within a narrow near-neutral range (0 to -7 mV) across all formulations (Figure 3-5c), with only minor variation observed across the design space. Small shifts were associated with changes in DSPC proportion, while cholesterol, SM-102, and DMG-PEG2k showed minimal influence.

Encapsulation efficiency varied with lipid composition (Figure 3-5d). Higher DSPC proportions were associated with reduced encapsulation efficiency, whereas higher cholesterol levels were associated with increased encapsulation efficiency. Higher SM-102 proportions were also associated with reduced encapsulation efficiency; however, as this reflects a mixture design, this trend represents the effect of increasing SM-102 relative to the other lipid components rather than its isolated contribution. DMG-PEG2k showed a limited, localised effect.

Protein expression varied non-linearly across the formulation space (Figure 3-5e). Higher DSPC proportions were associated with an increase in expression near the reference composition, followed by a reduction at higher levels. Higher cholesterol proportions were associated with increased expression across the design space. SM-102 exhibited a U-shaped relationship, while DMG-PEG2k showed a localised effect around the reference composition.

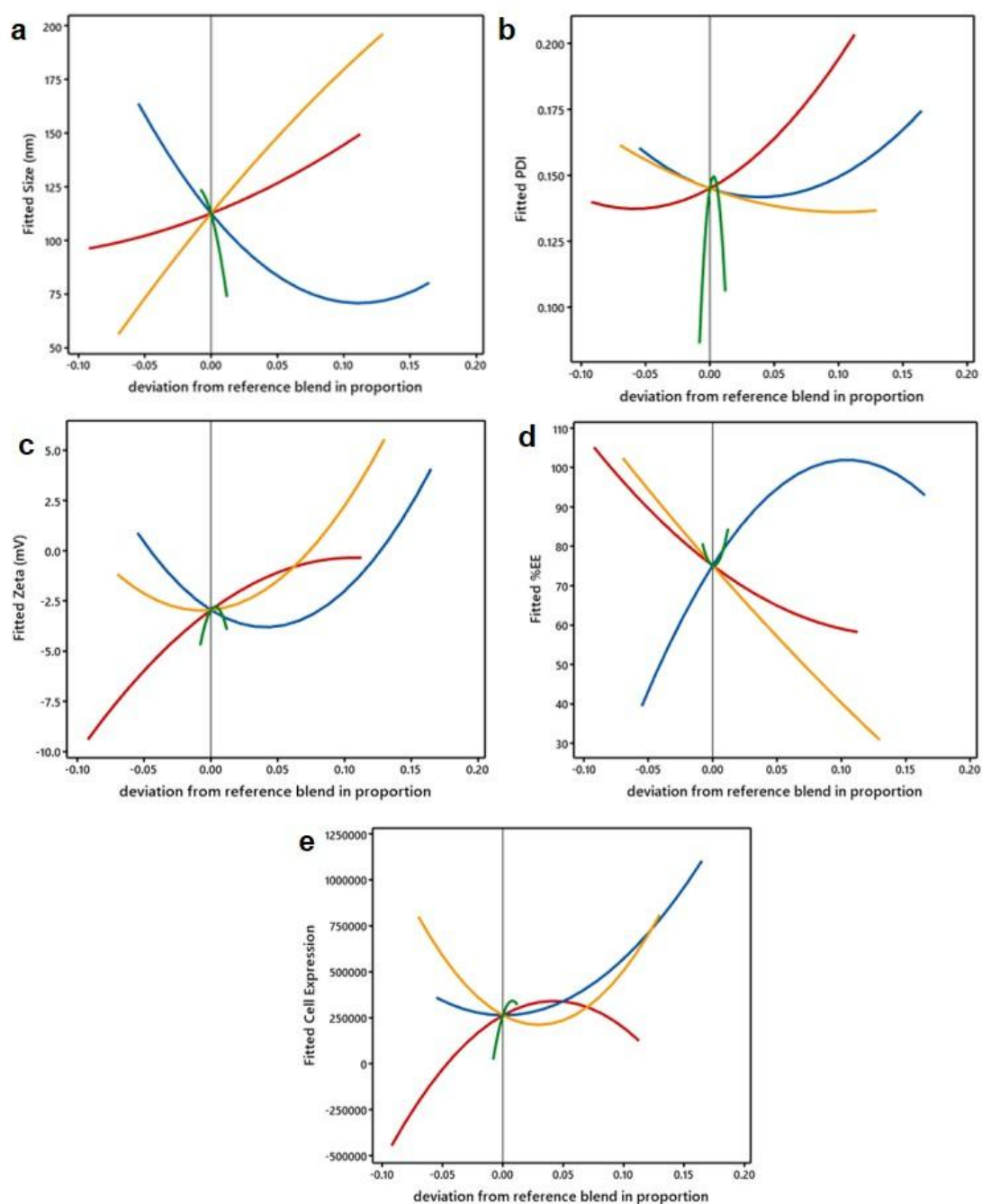


Figure 3-5 Cox Response Trace Plots for mDoE LNPs. The response trace plots demonstrate the relationship between size (a), PDI (b), zeta potential (c), %EE (d), and cell expression (Flux) (e) and changes in relative molar proportions of lipid components within the mixture. The grey line represents the reference blend which is indicated as the centroid of the vertices within the model at 18.78% DSPC, 43.06% cholesterol, 36.83% SM-102 and 1.33% DMG-PEG2K. Individual lipids are represented as coloured lines: DSPC (red), cholesterol (blue), SM-102 (yellow) and DMG-PEG2K (green). Cox Response Trace Plots were generated using Minitab.

3.4.6 Modelling the Characteristics of Manual DoE LNPs (mLNPs)

Data obtained from physicochemical characterisation and expression studies were used to generate predictive models for each output using Minitab. These models were visualised as contour plots to provide a spatial representation of the composition–response relationships identified in the Cox response trace plots.

Figure 3-6 presents contour plots for each response, with DSPC, cholesterol, and SM-102 represented on the ternary axes, while DMG-PEG2k was fixed at low (0.5%), medium (1.5%) and high (2.5%) molar ratios.

Figures 3-6a and 3-6b show the predicted effects of composition on particle size and PDI. Consistent with trends observed in the Cox response trace plots, higher DMG-PEG2k proportions were associated with smaller particle sizes and lower PDI. The largest particle sizes (dark green regions) were predicted in compositions characterised by lower cholesterol and DMG-PEG2k and higher DSPC proportions. Across all DMG-PEG2k levels, PDI remained below the acceptance threshold of 0.2.

Figures 3-6c–e shows the predicted effects of composition on zeta potential, encapsulation efficiency, and protein expression across the three DMG-PEG2k levels. The overall distribution of responses was similar across all PEG-lipid conditions, indicating that variation in DMG-PEG2k had limited impact on these outputs within the explored design space. Zeta potential (Figure 3-6c) remained within a narrow near-neutral range (approximately 0 to –10 mV), with only minor variation across compositional regions. Encapsulation efficiency (Figure 3-6d) was highest in regions characterised by increased cholesterol and lower SM-102 proportions, consistent across all PEG-lipid conditions. Protein expression (Figure 3-6e) showed a similar spatial distribution, with higher expression observed in regions corresponding to elevated encapsulation efficiency. These contour plots therefore provide a complementary visualisation of the composition–response relationships identified in Section 3.4.5

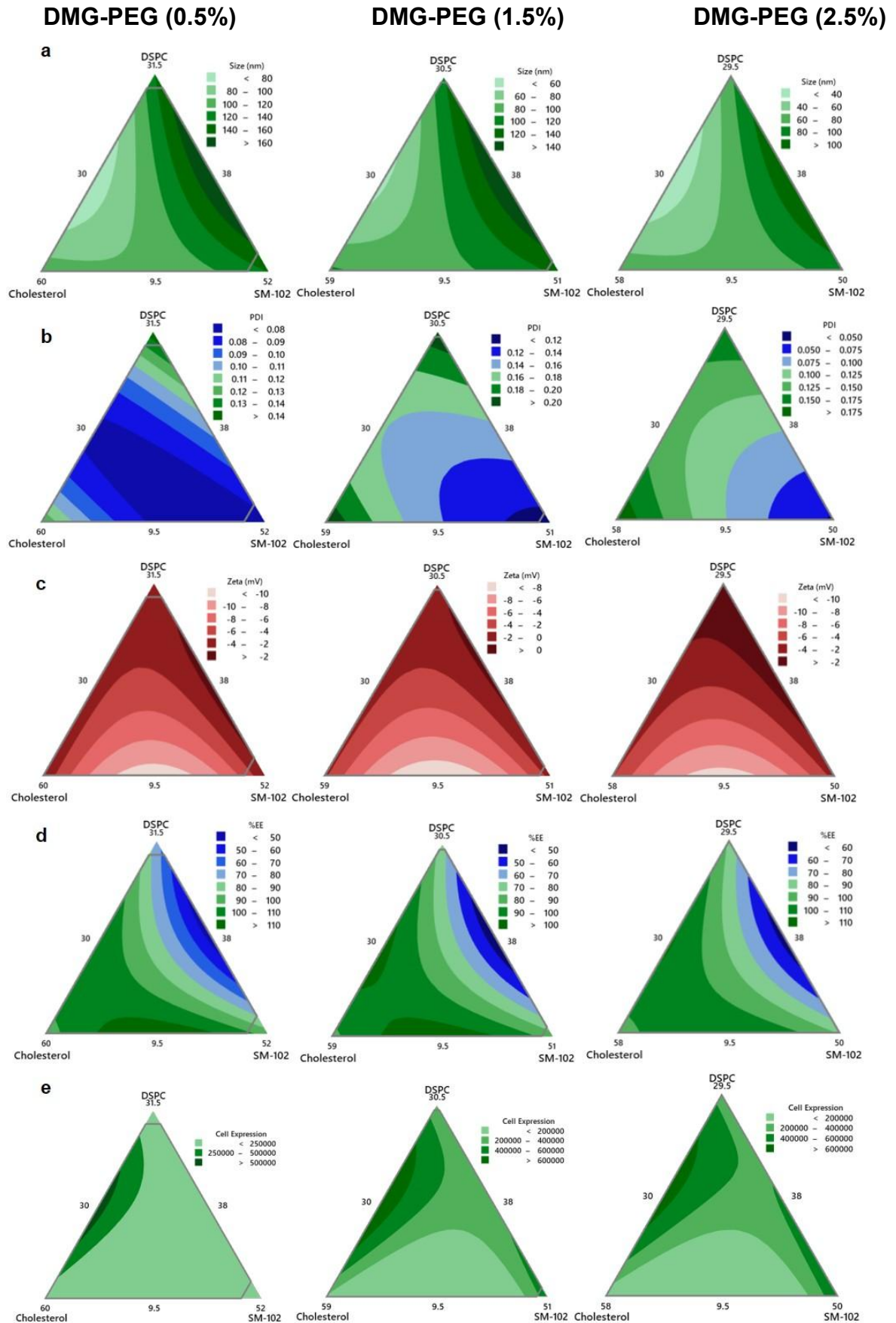


Figure 3-6 Contour Plots developed from the mLNP Model. Generated using Minitab, these contour plots demonstrate the relationship between lipid composition and (a) z-average diameter (nm), (b) polydispersity index, (c) zeta potential (mV), (d) encapsulation efficiency (%) and (e) in vitro protein expression in HEK-293 cells. The ternary axes represent DSPC, cholesterol, and SM-102, while DMG-PEG2k was held constant at low (0.5%), medium (1.5%) and high (2.5%) molar percentages. Changes in response magnitude are dictated by different colours with the points representing the highest molar percentage of the respective lipid, with the molar percentage decreases as you move down the axis with each area on the triangle representing a specific composition.

3.4.7 Validating mLNP DoE as a Predictive Model

The data were used to fit a regression model, enabling prediction of the CQAs for previously tested OFAT LNPs, thereby assessing the predictive capability of the mLNP model. Table 3-5 presents the predicted and actual values for OFAT LNPs, along with the prediction accuracy. Prediction accuracy was defined as values within 80 – 120% of the experimentally measured data.

Regression analysis was statistically significant for both mLNP size and polydispersity index (PDI) ($p < 0.05$); however, the models showed limited goodness of fit, with R^2 values of approximately 50% (Table 3-7). This is reflected in the predictive modelling of the OFAT LNPs (Table 3-5), where the model accurately predicted LNP1, LNP6, LNP8, and LNP9, but overestimated the size of LNP3 and LNP5. Additionally, the model did not accurately predict PDI.

The regression analyses for the remaining outputs (zeta potential, encapsulation, and *in vitro* expression) were not statistically significant ($p > 0.05$), with R^2 values ranging from 15% to 36% (Table 3-7). Accordingly, these models showed poor predictive performance for zeta potential and *in vitro* expression. However, the model accurately predicted encapsulation efficiency for LNP1, LNP6, and LNP8. Overall, the model demonstrated limited predictive capability and could not be robustly validated across the evaluated CQAs, prompting further investigation into whether improved process control could enhance model performance.

3.4.8 Composition-Response Relationships in Automated DoE LNPs (aLNPs)

To assess whether improved process control could enhance model performance, the DoE study was repeated using an automated microfluidic platform. The CQAs of aLNPs were analysed using the same methodology as for mLNPs, with the addition of *in vivo* evaluation and inclusion of the standard formulation as a

control (aLNP20). Results are shown in Figure 3-7, with corresponding data summarised in Figure 3-11.

Overall, aLNP formulations exhibited particle sizes <206 nm, PDI <0.2, zeta potentials between 0 and 17 mV, and encapsulation efficiencies >79%. Particle size trends were broadly consistent with those observed for mLNPs, increasing DSPC and SM-102 proportions were associated with larger particle sizes, while increasing DMG-PEG2k resulted in smaller particles (Figure 3-7a). However, in contrast to the manual model, increasing cholesterol was associated with increased particle size. The largest particles were observed for aLNP11 (~205 nm), which combined reduced DMG-PEG2k, increased cholesterol, and reduced SM-102 relative to the reference formulation (Figure 3-11). Polydispersity index remained low across all formulations, with minimal variation observed (Figure 3-7b).

Zeta potential values were slightly more positive overall compared to mLNPs, ranging from 0 to 17 mV (Figure 3-7c). Variation across the design space was limited, with only minor shifts observed. Increasing DSPC and cholesterol were associated with small reductions in zeta potential, while SM-102 showed a modest decreasing trend at higher proportions. DMG-PEG2k exhibited a localised effect around the reference composition. Encapsulation efficiency remained strongly dependent on lipid composition (Figure 3-7d). Higher encapsulation efficiencies were observed in regions with increased cholesterol and reduced SM-102, consistent with the manual model.

Protein expression exhibited non-linear behaviour across both *in vitro* (Figure 3-7e) and *in vivo* (Figure 3-7f) models. In both systems, increasing cholesterol was associated with increased expression, while higher DSPC proportions were associated with reduced or marginally affected expression at higher compositions *in vitro*. DMG-PEG2k showed a localised effect around the reference composition. Differences were observed for SM-102: it displayed a U-

shaped relationship *in vitro*, whereas *in vivo* expression increased with decreasing SM-102.

Corresponding experimental data are shown in Figure 3-11d–e. Two formulations, aLNP20 (standard composition) and aLNP9, demonstrated concordance between *in vitro* and *in vivo* expression. In contrast, aLNP4, which showed the highest *in vivo* expression, exhibited low *in vitro* expression and lower encapsulation efficiency.

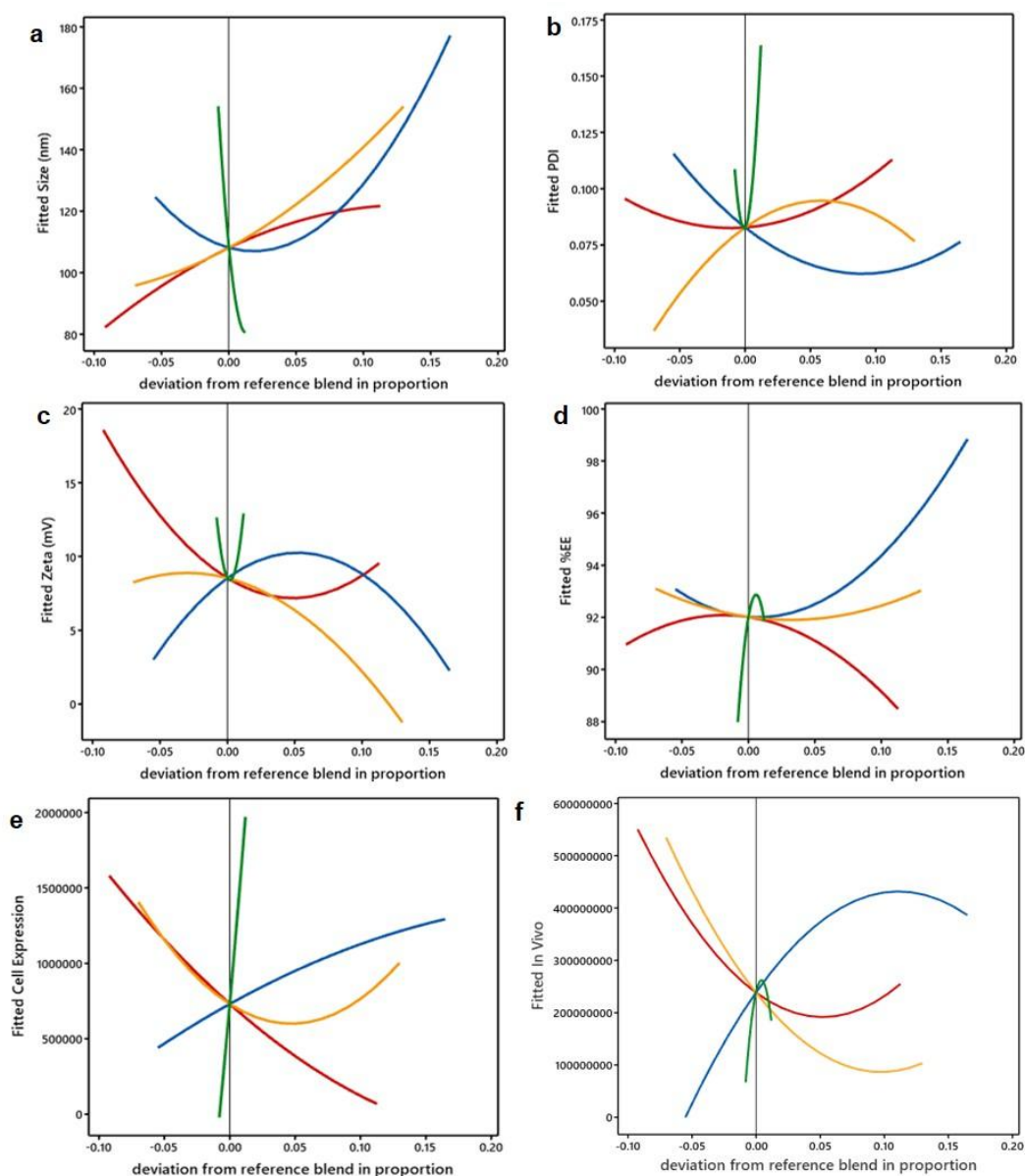


Figure 3-7 Cox Response Trace Plots for aDoE LNPs. The response trace plots demonstrate the relationship between size (a), PDI (b), zeta potential (c), %EE (d), cell expression (Flux) (e), and in vivo protein expression (Total Flux [p/s]) (f) and changes in the relative molar proportions of the lipid components within the mixture. The grey line represents the reference blend which is indicated as the centroid of the vertices within the model at 18.78% DSPC, 43.06% cholesterol, 36.83% SM-102 and 1.33% DMG-PEG2K. Individual lipids are represented as coloured lines: DSPC (red), cholesterol (blue), SM-102 (yellow) and DMG-PEG2K (green). Cox Response Trace Plots were generated using Minitab.

3.4.9 Modelling the Characteristics of Automated DoE LNPs (aLNPs)

Contour plots were generated to visualise the spatial distribution of composition–response relationships across the design space (Figure 3-8),

complementing the directional trends identified in the response trace plots (Figure 3-7). Figures 3-8a and 3-8b highlight distinct compositional regions associated with smaller and more homogeneous particles. These regions were consistently located at higher cholesterol proportions and reduced SM-102, and were maintained across all DMG-PEG2k levels, indicating limited dependence on PEG-lipid content. Zeta potential (Figure 3-8c) remained positive across the design space, with only moderate variation and no clearly defined compositional regions associated with substantially different surface charge. Encapsulation efficiency (Figure 3-8d) formed a well-defined high-encapsulation region at increased cholesterol and reduced SM-102, which was preserved across all DMG-PEG2k levels. *In vitro* protein expression (Figure 3-8e) exhibited greater spatial variability than other outputs, with multiple regions of increased expression observed across the design space. The location of these regions varied with DMG-PEG2k level, indicating sensitivity to PEG-lipid content. In contrast, *in vivo* protein expression (Figure 3-8f) showed a more consistent spatial distribution, with higher expression localised to a single compositional region characterised by reduced SM-102 and increased cholesterol. This region was maintained across all DMG-PEG2k levels.

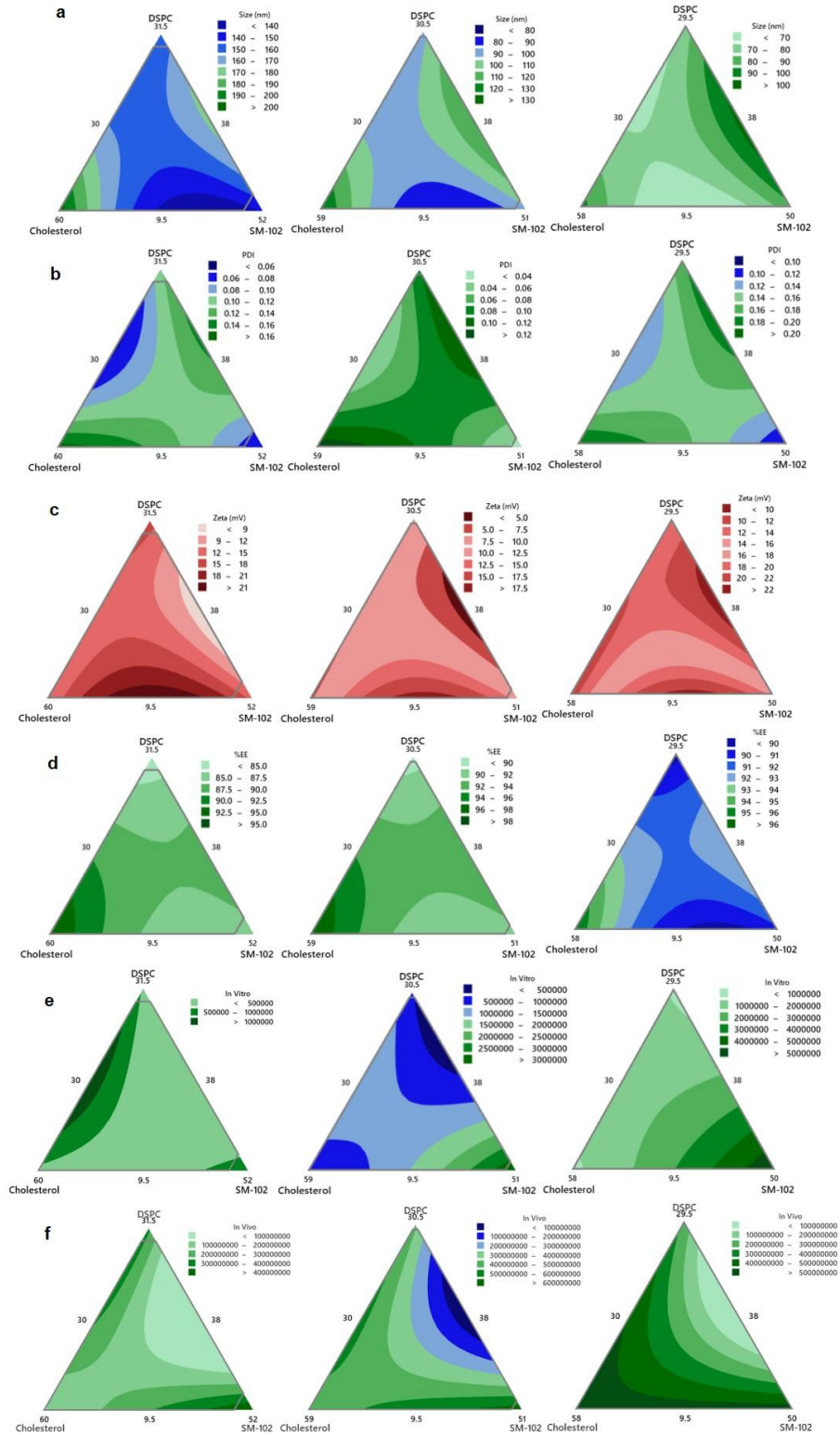


Figure 3-8 Contour Plots developed from the aLNP Model. Generated on Minitab, these contour plots demonstrate the 2D relationship between the lipids and (a) z-average diameter (nm), (b) polydispersity index, (c) zeta potential (mV), (d) encapsulation efficiency (%), (e) in vitro protein expression in HEK-293 cells and (f) in vivo protein expression in BALB/c mice. The X, Y and Z axis consist of DSPC, cholesterol and SM-102 while DMG-PEG2k was held constant at low (0.5%), medium (1.5%) and high (2.5%) molar percentages. Changes in response magnitude are dictated by different colours with the points representing the highest molar percentage of the respective lipid, with the molar percentage decreases as you move down the axis with each area on the triangle representing a specific composition.

3.4.10 Validating aDoE LNPs as a Predictive Model

Finally, the aLNP DoE formulations were used to fit a regression model, which could be used to analyse the previous OFAT samples and to generate prediction accuracy for a range of CQAs, as shown in Table 3-6. The regression model analysed from the aLNP DoE was statistically significant ($p < 0.05$) (Table 3-7) for four outputs: LNP size, PDI, %EE, and *in vitro* protein expression. Particle size was predicted with the highest accuracy ($R^2 = 81.74\%$) (Table 3-7), and predictions were accurate for LNP1, LNP6, LNP8, and LNP9. PDI and encapsulation efficiency showed moderate predictive performance ($R^2 = 48\%$ and 44% , respectively) (Table 3-7), with encapsulation efficiency accurately predicted for all formulations except LNP9. Finally, while *in vitro* protein expression has low accuracy for all LNPs, the model was able to predict which formulations would be the best-performing LNPs. In contrast, models for zeta potential and *in vivo* protein expression were not statistically significant ($R^2 = 5\%$ and 43% , respectively) (Table 3-7). However, the *in vivo* model could accurately predict *in vivo* expression for LNP1, LNP3 and LNP8 (Table 3-6). Overall, predictive performance improved compared to the manual DoE model; however, predictive power remained limited for both physicochemical CQAs (particularly for particle size and encapsulation efficiency) and biological outputs, and the aLNP DoE model could not be robustly validated across all CQAs.

3.5 Discussion

3.5.1 Revisiting the Lipid Ratio: Insights from OFAT and DoE

Despite the clinical success of nucleic acid–LNP vaccines, continued optimisation of formulation design remains essential as applications expand beyond hepatic delivery and into increasingly complex biological contexts. Current LNP formulations are typically based on established lipid ratios derived from clinically successful systems; however, these ratios are often adopted as fixed formulations rather than systematically optimised across broader compositional spaces [189, 190].

While OFAT approaches enable interrogation of individual variables [92, 105], they do not resolve interaction effects within multicomponent formulations.

This study revisited the commonly used lipid ratio in LNP formulations by directly comparing OFAT and DoE approaches to determine whether the standard formulation represents an optimal or merely acceptable composition.

The OFAT analysis indicated that the standard formulation is relatively robust, with most formulations maintaining acceptable CQAs within the explored compositional space (Figures 3-2 and 3-3). However, DoE revealed that this ratio represents only one point within a broader compositional landscape, with multiple regions producing comparable or improved physicochemical properties and expression (Figures 3-5 to 3-8). These findings indicate that LNP performance is governed by compositional balance rather than a single fixed lipid ratio.

3.5.2 OFAT Defines Formulation Boundaries but Not Optimal Ratios

For the OFAT formulations, molar ratios were derived from the standard LNP composition, with only the structural and ionisable lipids systematically varied. The OFAT study defined the boundaries of acceptable formulation space but provided limited insight into optimal lipid ratios.

Across the OFAT dataset, most formulations maintained CQAs within acceptable ranges (Figure 3-2), indicating that the standard formulation is relatively robust to compositional variation. However, increasing DSPC and removing cholesterol led to greater heterogeneity and reduced expression (Figures 3-2 and 3-3). These observations are consistent with the established role of cholesterol in stabilising lipid packing and supporting LNP self-assembly [106].

Variation in DSPC and SM-102 produced comparatively modest and largely directional effects across CQAs within the explored compositional space. Previous studies have demonstrated that ionisable lipid content influences lipid organisation and membrane behaviour [191]; however, within the constrained

OFAT space explored here, these effects were not sufficient to define optimal formulation compositions.

DSPC and cholesterol showed opposing trends in expression (Figure 3-3), with reduced cholesterol associated with lower efficacy. These findings are consistent with reports that phospholipid–cholesterol interactions contribute to membrane organisation and LNP functionality [192, 193]. Overall, these data demonstrate that OFAT is effective for identifying formulation limits and directional trends but does not resolve interaction effects or define optimal lipid ratios within the broader compositional space.

3.5.3 DoE Reveals Composition-Response Relationships Beyond the Standard Ratio

In contrast to OFAT, DoE analysis revealed non-linear and interaction-driven behaviour across the formulation space, enabling identification of compositional regions associated with favourable CQAs and expression profiles (Figures 3- 5 to 3-8).

Across both manual and automated DoE models, cholesterol and SM-102 emerged as dominant and opposing drivers of formulation performance. Increasing cholesterol was consistently associated with improved encapsulation efficiency and protein expression, whereas increasing SM-102 had a more variable impact on these outputs within the explored mixture space. This highlights that formulation performance is governed by relative lipid balance rather than the contribution of any single component.

DMG-PEG2k primarily influenced particle size and PDI, with higher proportions associated with reduced particle diameter, consistent with previous reports describing steric stabilisation effects of PEGylated lipids [166]. However, its influence on biological performance was more localised and dependent on the surrounding lipid composition. DoE also revealed component-dependent trade-

offs between physicochemical attributes and biological performance that were not apparent from OFAT analysis alone.

Comparison of manual and automated DoE models highlighted both consistent and divergent trends. While the influence of SM-102 and DMG-PEG2k was broadly consistent across models, the direction and magnitude of the effects of DSPC and cholesterol differed between models (Figures 3-5 to 3-8), indicating sensitivity to processing conditions.

These differences were observed despite comparable CQA ranges between models, suggesting that formulation–process interactions contribute to the observed variability. Given that mixing conditions are known to influence LNP formation and properties [121], these findings indicate that manufacturing execution can alter apparent composition–response relationships.

Despite improved consistency in the automated system, predictive modelling remained limited. While automation improved model fit for particle size and encapsulation efficiency, prediction of biological outputs, particularly *in vivo* expression, remained poor (Tables 3-5 and 3-6). This demonstrates that although DoE effectively maps the formulation space and identifies optimal compositional regions, it does not necessarily yield robust predictive models for complex biological outcomes.

3.5.4 Cross-Method Comparison of Lipid-Response Relationships

To further compare lipid–response relationships across formulation strategies, correlation heat maps were used to compare lipid–response relationships across the OFAT, mDoE, and aDoE datasets (Figure 3-9).

The OFAT heat map exhibited high-magnitude correlations between individual lipids and measured outputs; however, these relationships were confined to the limited compositional space explored and primarily reflected local, directional effects (Figure 3-9a).

In contrast, both DoE-based heat maps showed more distributed and lower-magnitude correlations across lipid components, consistent with interaction-driven behaviour within a multicomponent formulation space. Notably, the DoE datasets exhibited broader contributions across multiple lipid components, rather than the dominant single variable observed in OFAT, consistent with previous reports highlighting the multivariate nature of LNP formulation behaviour [83]. Subtle differences between the mDoE (Figure 3-9b) and aDoE (Figure 3-9c) heat maps suggest that manufacturing execution influences the apparent strength and direction of lipid–response relationships. Taken together, these data indicate that OFAT yields strong apparent lipid–CQA correlations within a restricted compositional space, whereas DoE reveals more distributed relationships consistent with interaction-driven behaviour.

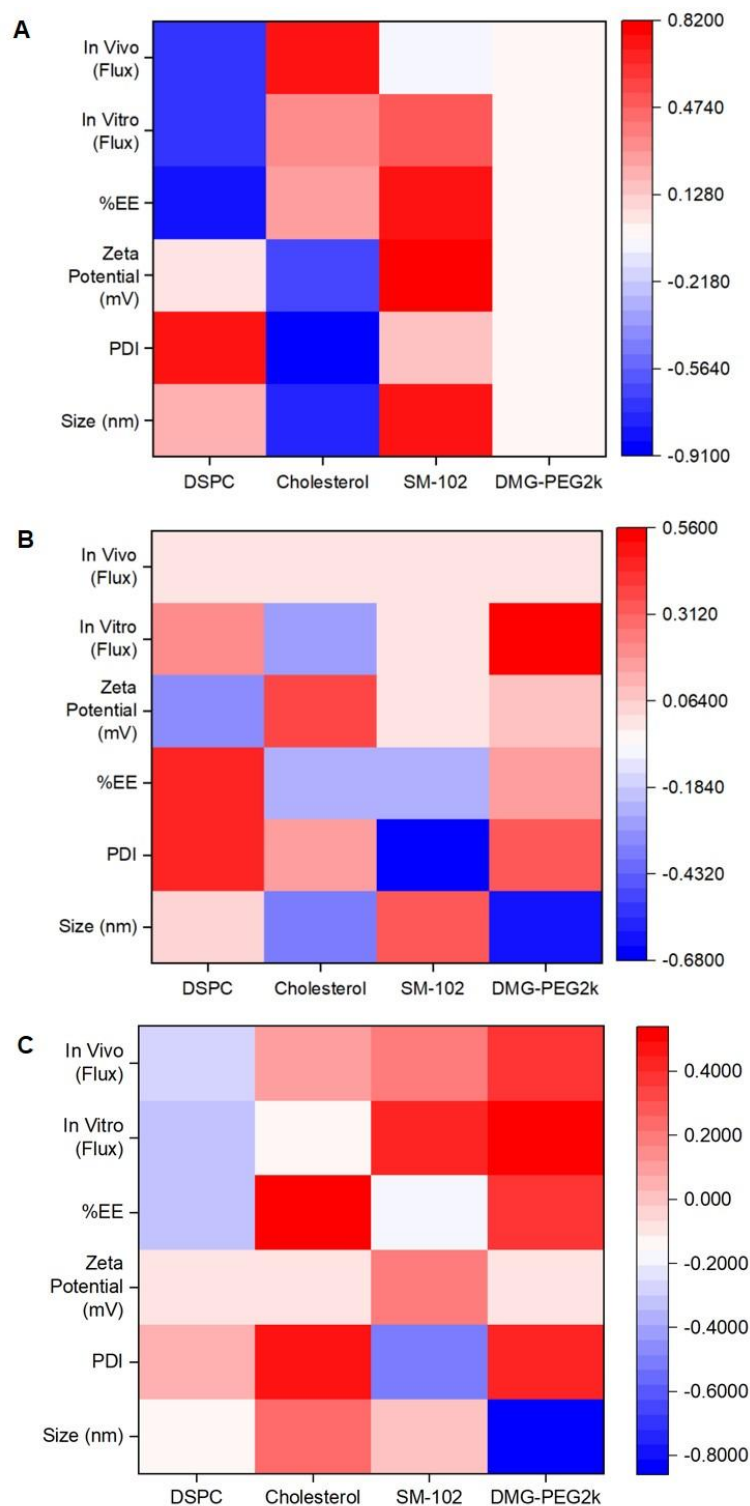


Figure 3-9 Lipid–response correlation heat maps comparing formulation strategies. Heat maps show correlation coefficients between lipid molar ratio components and measured physicochemical properties and in vitro and in vivo expression for (a) OFAT, (b) manually executed DoE (mDoE), and (c) automated DoE (aDoE) datasets. Colour intensity represents the strength and direction of correlation (red, positive; blue, negative). Heat maps are intended to enable comparative visualisation of lipid–response relationships across formulation strategies rather than direct quantitative prediction. Models were generated using Origin Pro 2022.

3.5.5 Implications for Moving Beyond a Fixed Ratio

Previous studies have demonstrated the value of DoE approaches for interrogating lipid composition and process effects in LNP systems [83, 140, 194]. However, these approaches are often applied without direct comparison to traditional one-factor-at-a-time (OFAT) strategies. The present study addresses this gap by directly comparing OFAT and DoE within a consistent formulation framework, demonstrating that while OFAT is effective for defining formulation boundaries and confirming the robustness of the standard lipid ratio, DoE provides broader insight into non-linear, interaction-driven behaviour across the formulation space.

Importantly, despite these advantages, DoE did not yield robust predictive models for biological outcomes, even when implemented using an automated platform. This highlights a key limitation of composition-based modelling approaches, indicating that formulation optimisation cannot rely on lipid ratio alone, but must also consider process conditions and biological context. Together, these findings support a shift from fixed-ratio formulations towards region-based optimisation strategies, in which multiple compositions within a defined design space may achieve comparable performance and emphasise the need to integrate formulation design with process control and biological evaluation.

Overall, the present work demonstrates that OFAT and DoE provide fundamentally different but complementary insights into LNP formulation behaviour, with OFAT suited to identifying directional effects and formulation boundaries, and DoE enabling systematic exploration of interaction effects and non-linear trade-offs. For the field, this highlights the need to move beyond inherited formulation conventions and adopt integrated optimisation strategies, with further gains expected through alignment with Quality-by-Design frameworks and advanced data-driven modelling approaches [195].

3.6 Conclusions

This study demonstrates that the conventional lipid ratio represents a robust starting point but not an optimal formulation within a broader, flexible design space. Multiple compositions achieved comparable physicochemical properties and biological performance, highlighting that LNP behaviour is governed by compositional balance rather than a single fixed ratio. While DoE enabled exploration of interaction effects and formulation trade-offs beyond OFAT, predictive performance remained limited, particularly for biological outputs, and was influenced by both formulation and processing conditions. These findings highlight a disconnect between formulation mapping and predictive capability. Moving forward, LNP optimisation should shift from fixed ratios to design-space-driven strategies that integrate composition, process, and biological context.

3.7 Supplementary Information for Publication

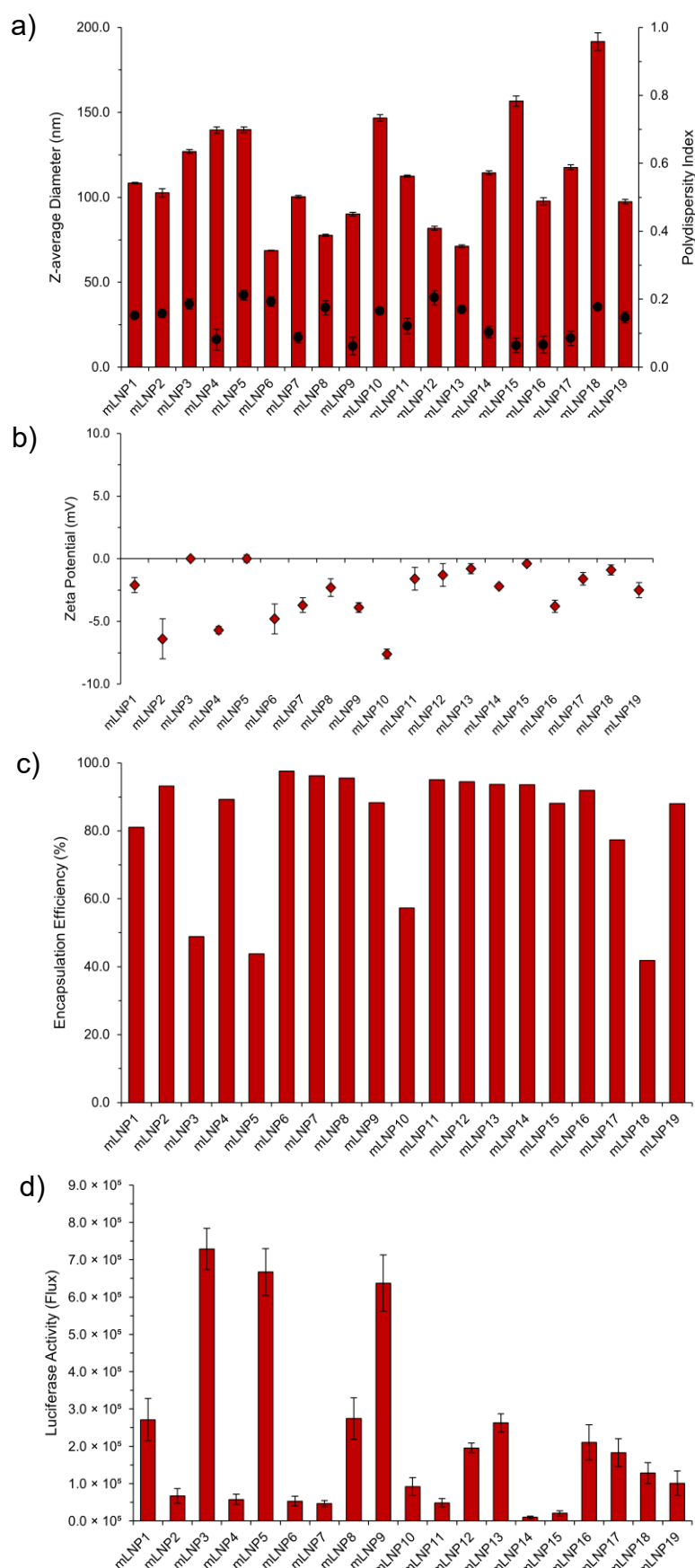
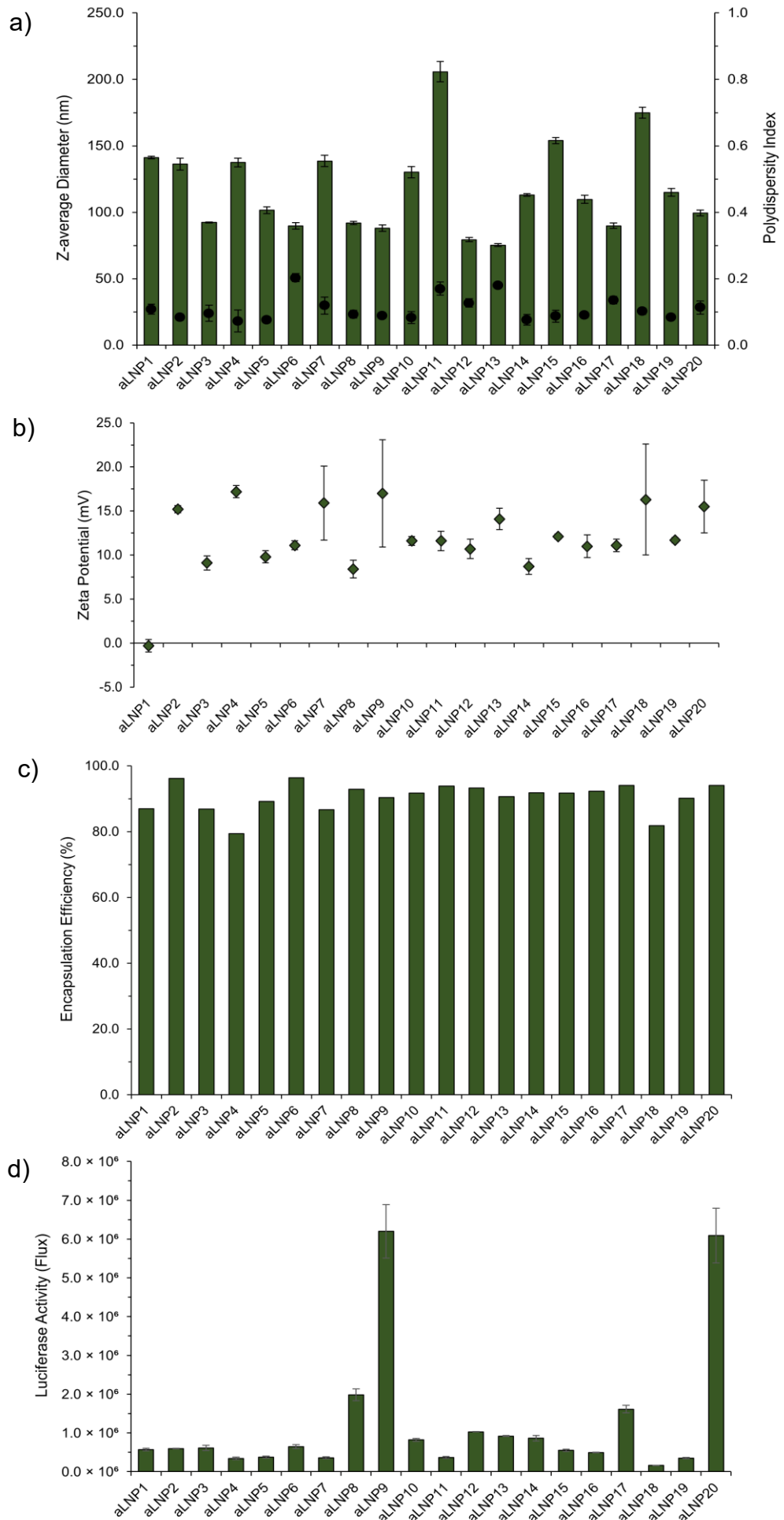


Figure 3-10 CQAs for mDoE LNP Formulations. (a) represents the z-average diameter (nm) (bars) and polydispersity index (circles), while (b) and (c) represent the zeta potential (mV) and 158 encapsulation efficiency (%) respectively. (d) shows the protein expression (Flux) at a treatment concentration of 2 μ g/mL mRNA for mDoE formulations. Data (excluding encapsulation efficiency) are presented as mean $\pm$ SD of three technical replicate measurements ($n = 1$).



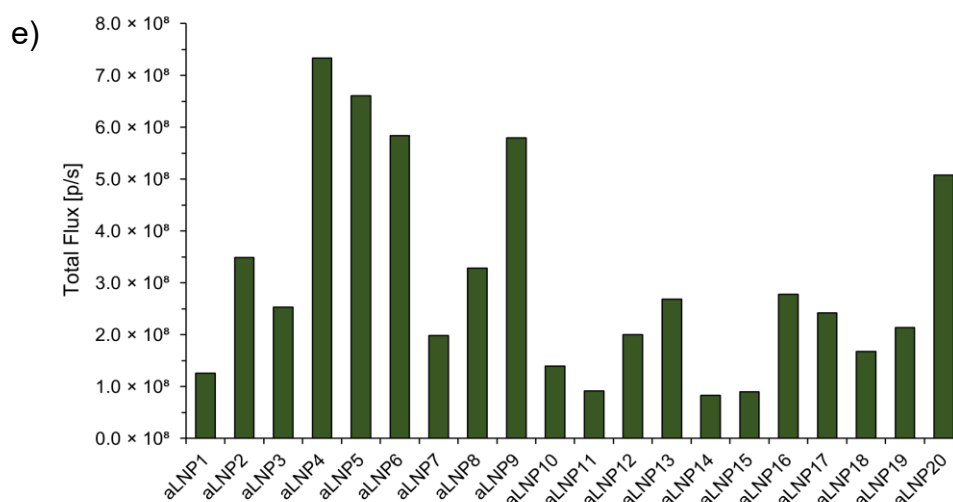


Figure 3-11 CQAs for aDoE LNP Formulations. (a) represents the z-average diameter (nm) (bars) and polydispersity index (circles), while (b) and (c) represent the zeta potential (mV) and encapsulation efficiency (%) respectively. (d) shows the protein expression (Flux) in HEK-293 cells at a treatment concentration of 2 $\mu\text{g/mL}$ mRNA for aDoE formulations while (e) represents the protein expression (total flux [p/s]) at the injection site of BALB/c mice 6 hours post intramuscular injection with aDoE formulations. aLNP20 represents the standard molar ratio. Data (excluding encapsulation efficiency and in vivo expression) are presented as mean $\pm$ SD of three technical replicate measurements ($n = 1$).

Table 3-5 . Predicted and Actual CQAs from OFAT LNPs and Predictive mLNP Model.

	Z-average Diameter (nm)			Polydispersity Index			Zeta Potential (mV)			Encapsulation Efficiency (%)			Cell Expression (Flux)		
	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy
LNP1	123.2	110.0	112.0	0.081	0.105	77.1	-3.6	1.8	-200.0	83.2	94.4	88.1	242848	83087484.2	0.3
LNP3	149.0	111.6	133.5	0.092	0.121	76.0	-1.0	1.0	-100.0	59.4	74.6	79.6	448939	5957067.5	7.5
LNP5	172.9	99.8	173.2	0.102	0.318	32.1	1.4	3.2	43.8	37.4	69.3	54.0	639573	35406150.8	1.8
LNP6	110.2	111.2	99.1	0.075	0.057	131.6	-4.9	0.7	-700.0	95.1	97.3	97.7	139803	74111959.2	0.2
LNP8	109.9	91.2	120.5	0.174	0.113	154.0	-1.3	0.4	-325.0	73.5	77.2	95.2	298430	4331792.5	6.9
LNP9	103.2	92.7	111.3	0.221	0.121	182.6	-0.1	-1.7	68.6	68.6	41.8	164.1	326221	20285874.4	1.6

Table 3-6 Predicted and Actual CQAs from OFAT LNPs and Predictive aLNP Model

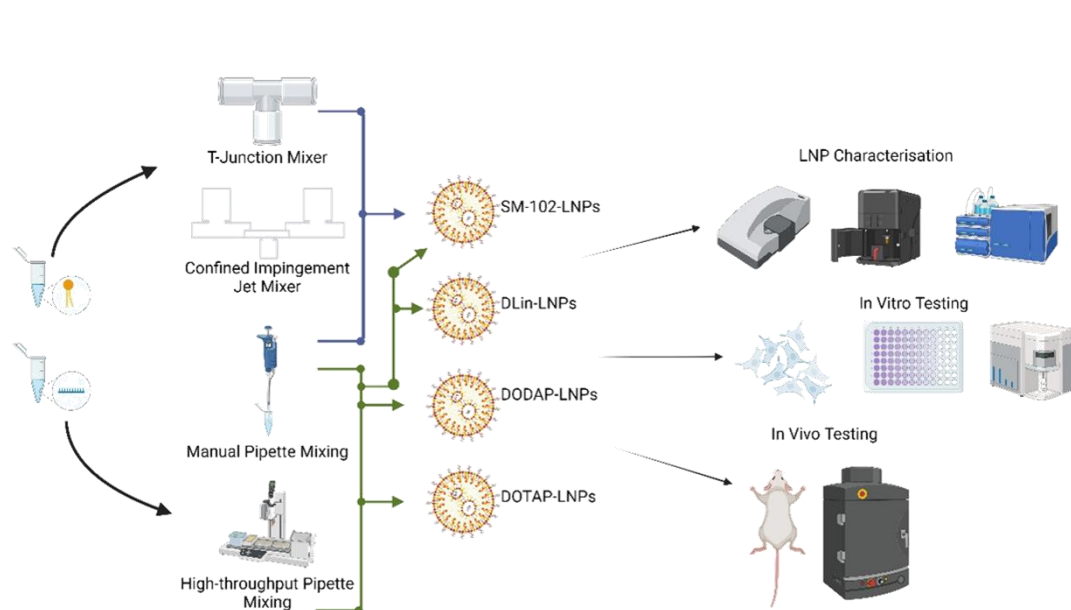
	Z-average Diameter (nm)			Polydispersity Index			Zeta Potential (mV)			Encapsulation Efficiency (%)			Cell Expression (Flux)			In vivo Expression (Flux)		
	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy	Predicted	Actual	Accuracy
LNP1	105.1	110	95.5	0.081	0.105	77.1	13.1	1.8	727.8	89.9	94.4	95.2	2411289	83087484.2	2.9	393766639	427752520	92.1
LNP3	76.8	111.6	68.8	0.043	0.121	35.5	13.4	1	1340.0	82.5	74.6	110.6	2251148	5957067.5	37.8	312387166	328152520	95.2
LNP5	50.5	99.8	50.6	0.008	0.318	2.5	13.7	3.2	428.1	75.7	69.3	109.2	2103019	35406150.8	5.9	237111153	25254186.67	938.9
LNP6	119.3	111.2	107.3	0.099	0.057	173.7	13	0.7	1857.1	93.5	97.3	96.1	2491359	74111959.2	3.4	434456376	278877520	155.8
LNP8	106.4	91.2	116.7	0.115	0.113	101.8	11	0.4	2750.0	88.7	77.2	114.9	478310	4331792.5	11.0	252570419	281874186.7	89.6
LNP9	107.1	92.7	115.5	0.132	0.121	109.1	9.9	-1.7	-582.4	88.1	41.8	210.8	-488180	20285874.4	-2.4			

Table 3-7 Model Fit Analysing of mDoE and aDoE Regression Model used for Predictions in Table S1 and S2.

	mDoE Model Fit		aDoE Model Fit	
	R ²	p-value	R ²	p-value
Size	51.62	0.011	81.74	<0.001
Polydispersity Index	51.27	0.011	47.61	0.019
Zeta Potential	21.72	0.284	4.66	0.864
Encapsulation Efficiency	16.27	0.432	44.27	0.029
In vitro	35.69	0.078	54.25	0.007
In vivo	N/A	N/A	18.05	0.379

Chapter 4: Low-Cost Microfluidic Mixers: Are They up to the Task?

Type of Publication	☒ Published article ☐ Accepted for publication ☐ Submitted for publication ☐ Drafted for publication/application notes ☐ Not applicable
Citation Details (only applicable if published)	Forrester, J., Davidson, C. G., Blair, M., Donlon, L., McLoughlin, D. M., Obiora, C. R., Stockdale, H., Thomas, B., Nutman, M., Brockbank, S., Rattray, Z., & Perrie, Y. (2025). Low-Cost Microfluidic Mixers: Are They up to the Task? <i>Pharmaceutics</i> , 17(5), 566. https://doi.org/10.3390/pharmaceutics17050566
Chapter Inclusion Details	☒ This chapter is a reproduction of the published article with minor wording changes. ☐ This chapter contains minor revision of the original publication ☐ This majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	Conceptualization, Y.P., J.F. and D.M.M.; data curation, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R.; formal analysis, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R.; funding acquisition, Y.P., L.D., D.M.M., S.B. and Z.R.; investigation, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R.; methodology, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R.; project administration, Y.P., L.D., D.M.M., S.B. and Z.R.; resources, Y.P., L.D., D.M.M., S.B. and Z.R.; supervision, Y.P., L.D., D.M.M., S.B. and Z.R.; validation, Y.P., J.F., C.G.D., M.B., D.M.M., C.R.O., H.S., B.T. and M.N.; visualization, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R.; writing—original draft, Y.P., J.F. and C.G.D.; writing—review and editing, Y.P., J.F., C.G.D., M.B., L.D., D.M.M., C.R.O., H.S., B.T., M.N., S.B. and Z.R. All authors have read and agreed to the published version of the manuscript. We would like to acknowledge Nima Hojat and Bradley Brown for automation scripting and workflow development. We also acknowledge James Streetley, Debakshi Millick, and Gonzalo Cebrero Acuna at the Scottish Centre for Macromolecular Imaging (SCMI) for assistance with cryo-EM experiments and access to instrumentation.



4.1 Abstract

Background/Objectives: Microfluidic mixing has become the gold standard procedure for manufacturing nucleic acid lipid-based delivery systems, offering precise control over critical process parameters. The choice and design of microfluidic mixers are often seen as a key driving force affecting the critical quality attributes of the resulting lipid nanoparticles (LNPs). **Methods:** This study aimed to evaluate LNPs manufactured using two low-cost microfluidic mixers alongside manual mixing (pipette mixing (PM)), followed by characterization studies using orthogonal analytics as well as expression studies to establish whether low-cost microfluidic manufacturing methods are suitable for bench-scale and high-throughput research. **Results:** The results show that all manufacturing methods can produce LNPs with sizes ranging between 95 and 215 nm with high encapsulation (70–100%), and enhanced analytics showed variations between the LNPs produced using the different mixers. Despite these differences, pipette mixing production of LNPs demonstrated its application as a high-throughput screening tool for LNPs, effectively distinguishing between different formulations and predicting consistent expression patterns both *in vitro* and *in vivo*. **Conclusions:** Overall, these results validate the use of low-cost

microfluidic mixers without compromising the efficiency and integrity of the resulting LNPs. This study supports the increased accessibility of small-scale LNP manufacturing and high-throughput screening.

4.2 Introduction

Traditionally, lipid nanoparticles and other nanoparticle delivery systems were commonly manufactured using complex techniques, often resulting in large particles requiring additional steps to reduce size, making these techniques hard to scale up and costly to maintain [112]. As nanoparticle technology has advanced, so too have manufacturing methods. In an effort to create user-friendly, cost-effective and scalable nanoparticle manufacturing methods, microfluidic mixing was developed. Microfluidic mixing can briefly be described as the mixing of two or more fluids within a microscale environment [196], with advantages including higher encapsulation efficiencies, the possibility to incorporate both lipophilic and hydrophilic drugs, and an overall decreased cost and time constraint [20, 109]. Microfluidics results in nanoparticle formation in a single-step process involving the mixing of an aqueous phase (commonly including the moiety to be entrapped dissolved in buffer, e.g., mRNA in the case of LNPs) with a lipid phase (dissolved in solvent, e.g., ethanol). Indeed, microfluidics is now routinely involved in LNP manufacture, and Pratsinis et al. demonstrated using a Design of Experiments (DoE) approach that when compared to solvent injection methods, microfluidics resulted in small LNPs with a more homogenous population [194]. Manufacturing using microfluidic devices can involve either active mixing (requiring external forces, e.g., vibration or electrical forces) or passive mixing (e.g., cross-flow or flow-focusing), which will additionally have variations in internal structures and diameters of mixers. This, in turn, can influence the critical quality attributes (CQAs) of the resulting LNP formulations [120, 121, 169]. The degree of mixing can also be influenced by the channel length and contact surface area of the liquid/liquid interface [169].

One of the earliest and easiest to use microfluidic mixer designs is a standard T-junction mixer, which typically involves the aqueous and lipid phases being injected directly into each other at high speeds, resulting in the formation of LNPs. This method often enables precise control over size; however, it requires increased waste volumes to allow for fluid mixing equilibration, making it less suitable for small-scale research. Additionally, the high forces generated in these mixers can disrupt droplet formation at the interface, potentially affecting LNP uniformity and stability [169, 197]. Despite this, T-junction mixers have demonstrated significant clinical success, with Pfizer BioNTech manufacturing their COVID-19 vaccine using a proprietary T-mixer design [198]. A second common design, known as impinging jet mixers, features a set-up in which fluids are constricted and directed toward each other at high speeds, resulting in nanoprecipitation. These mixers can be particularly useful when dealing with higher viscosity liquids. However, similar to T-mixers, these mixers are subject to high shear forces [121]. Despite these challenges, impinging jet mixers help overcome the complexities of fluidic control through fewer fluid channels and a simpler design [120]. As LNP systems have gained popularity, the demand for precise control over LNP CQAs, coupled with enhanced scalability, has increased. Mixers that excel in these parameters often harness chaotic flow, differing from traditional methods by incorporating grooves or advections that disrupt laminar flow [20, 121]. One example of a chaotic flow mixer is the staggered herringbone mixer (SHM), which contains two inlet channels flowing into a mixer with 'v' shaped advections, causing transverse flow and enhancing the mixing of the two fluids. Another example is the toroidal mixer (TM), which causes fluids to wrap around each other due to circular flow path structures [121, 169, 199]. Despite their advantages, industry-leading chaotic mixers are often expensive, rely heavily on single-use plastics, and are not the only viable option for producing small, homogenous LNP populations. Simplified schematic representations of these microfluidic mixers are shown in Figure 4-1; however,

these mixer designs encompass only a small portion of the diverse range of available mixing methods.

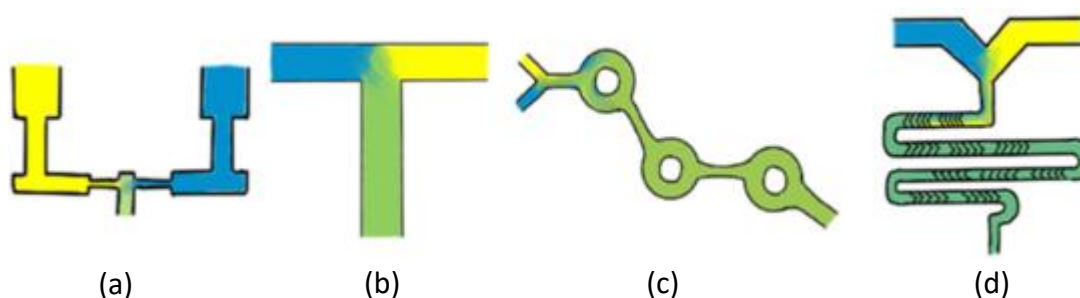


Figure 4-1 A simplified schematic of the common microfluidic mixers. Representations of the flow in common microfluidic mixers: (a) confined impingement jet mixer, (b) T-junction mixer, (c) toroidal mixer, and (d) staggered herringbone mixer. Image prepared using Sketchbook v6. 1.

Overall, different mixer designs can produce LNPs with varied CQAs, making it essential to understand how each mixer type influences nanoparticle characteristics to successfully select the most appropriate system. Beyond mixer design, the internal size of the mixer also plays a crucial role in LNP formation. For example, Maeki et al. conducted a study in which they designed two chaotic advection mixers with different channel depths and demonstrated that, for a liposomal formulation, a larger channel depth resulted in higher mixing efficiency [122]. Additionally, passive and active mixers can be combined to refine process parameters further. For instance, Peng et al. coupled ultrasonic microreactors with either co-flow or confined impingement jet mixers to manufacture liposomal formulations, illustrating the potential of hybrid approaches [200]. Furthermore, key process parameters, such as the speed at which the fluids are delivered (total flow rate; TFR) and the ratio at which the fluids are delivered (flow rate ratio; FRR), are vital in controlling the CQAs of LNPs. For example, McMillan et al. and Roces et al. both demonstrated that varying FRRs offered a precise method for controlling LNP size [19, 105].

Here, we briefly highlighted a small portion of the extensive diversity of microfluidic mixer design. This study aims to determine whether low-cost,

reusable microfluidic mixers can produce LNPs within a standard target CQA range with high payload entrapment and preclinical efficacy to allow for the low-cost and rapid screening of LNP formulations. To do this, two microfluidic mixing devices, a T-junction mixer (TM) and a confined impingement jet mixer (IJM), both equipped with automated syringe drivers, alongside a manual mixing process (pipette mixing (PM)), were used to manufacture LNPs. These LNPs were then subjected to orthogonal analysis techniques and preclinical *in vitro* and *in vivo* testing. A high throughput pipette mixing robot was also employed to assess PM as an LNP screening and optimization platform. Overall, this study aimed to establish if low-cost microfluidic mixers can reliably and reproducibly produce LNPs suitable for screening and testing of LNPs.

4.3 Materials and Methods

4.3.1 Materials

The ionizable lipids Heptadecan-9-yl 8-((2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino) octanoate (SM-102) and (6Z, 9Z, 28Z, 31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (D-Lin-MC3-DMA) were purchased from Broadpharm (San Diego, CA, USA). 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000), 1,2-distearoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) were acquired from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, polyadenylic acid (poly A), heat-inactivated fetal bovine serum (FBS) and HPLC-grade methanol were purchased from Merck Life Science UK Ltd. (Gillingham, UK). Ethanol was purchased from Suppelco (Bellefonte, PA, USA). Sodium citrate buffer, Tris-HCL buffer, Triethylamine acetate buffer, propidium iodide, Trypan Blue, and the Quanti-iT RiboGreen RNA Assay kit were purchased from Thermo Fisher Scientific (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). EZ Cap™ Firefly Luciferase mRNA (5-moUTP) and EZ Cap™ Cy5 Firefly Luciferase mRNA (5-moUTP) were purchased from APEXbio (Houston, TX,

USA). CPI (Centre for Process Innovation, Sedgefield, UK) supplied Green Lantern mRNA. A Rabbit Reticulocyte Lysate kit, ONE-Glo™ Luciferase Assay System, and VivoGlo™ Luciferin were acquired from Promega Ltd. (Chilworth, UK). Dulbecco's Minimal Essential Media (DMEM), Minimal Essential Media (MEM), sodium pyruvate, penicillin/streptomycin, and TrypLE were purchased from Gibco Technologies. Ultrapure water was supplied through an in-house system. HEK-293 cells were purchased from American Tissue Culture Collection (ATCC) (CRL-1573).

4.3.2 Microfluidic Mixer Set-Up

The T-junction mixer (TM) consisted of a 3-port manifold T-junction (Darwin Microfluidics); aqueous and lipid phase inlets flow directly into each other while the LNP outlet is positioned perpendicularly. The impingement jet mixer (IJM) consisted of a 5-port confined impinging jet mixer (CIJM) (Holland Applied Technologies, Burr Ridge, IL, USA); 3 of the 5 ports were used with the aqueous and lipid phase inlets flowing down, then directly into each other, with the LNP outlet positioned perpendicularly for sample collection. Both microfluidic mixers were combined with World Precision Instrument AL-1010 syringe pumps, which were automated using the SyringePumpPro software v1.7.0.0 to allow for precise control over mixing speeds and ratios. Pipette mixing (PM) was accomplished using Gilson P1000 and P200 pipettes (Dunstable, UK).

4.3.3 Manufacture of Lipid Nanoparticles (LNPs)

The aqueous phase consisted of poly A, Fluc mRNA, Green Lantern mRNA, or Cy5-Fluc mRNA diluted in 50 mM pH 5 citrate buffer to account for an N/P ratio of 6. The lipid phase consisted of DSPC, cholesterol, ionizable/cationic lipid (SM-102, D-Lin-MC3-DMA, DODAP, or DOTAP) and DMG-PEG2000 at a molar ratio of 10:38.5:50:1.5% dissolved in absolute ethanol ($\geq 99.5\%$). LNPs were manufactured on the TM, IJM, or PM to achieve LNPs with a final payload concentration of 0.04 mg/mL and a final lipid concentration of 0.9 mg/mL. For TM

and IJM, total flow rates (TFRs) were maintained at 15 mL/min, while the flow rate ratio (FRR) was kept constant at 3:1 (aqueous/lipid) for all mixers.

4.3.4 Purification of LNPs

Subsequent LNPs were purified using either dialysis or spin column chromatography. Dialysis purification consisted of LNPs being dialyzed against 10 mM pH 7.5 Tris buffer for 4 h using dialysis tubing with a molecular weight cut-off of 14 kDa. Spin column purification was carried out with LNPs being diluted 1:40 with 10 mM pH 7.5 Tris buffer, and centrifugal filtration was carried out using Amicon Ultra-15 columns at 2000× g 4 °C for approximately 10 min or until the desired volume was achieved.

4.3.5 LNP Characterisation

The critical quality attributes (CQAs) of LNPs were monitored using Dynamic Light Scattering (DLS)/Electrophoretic Light Scattering (ELS). The z-average diameter (nm), polydispersity index, and zeta potential (mV) were measured using the Zetasizer Ultra (Malvern Panalytical, Ltd., Worcestershire, UK). The LNPs were diluted to a 0.1 mg/mL lipid concentration for analysis. Pre-purification measurements were diluted in citrate buffer (viscosity 1.28 cP and refractive index 1.47), post-purification measurements were diluted in Tris buffer (viscosity 1.0037 cP and refractive index 1.337), and zeta potential measurements were diluted in water (viscosity 0.8872 cP and refractive index 1.33).

Payload encapsulations were quantified using the Quanti-iT™ RiboGreen™ Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). High (0–1000 ng) and low (0–50 ng) curves were prepared using the encapsulated payload to quantify the encapsulated material from Triton-treated and non-Triton-treated wells. Briefly, samples were diluted to a concentration of 3 µg/mL payload and mixed with either 50 µL of 2% Triton-TE or 1× TE buffer before being incubated for 15 min at 37 °C. Following on, a 200× and 500× RiboGreen solution was added to the

Triton-treated and non-Triton-treated wells, respectively, to account for a 1:1 dilution. The fluorescence was then measured using the Glomax (Promega, Chilworth, UK) at excitation 475 nm and emission 500–550 nm.

4.3.6 Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) was conducted using the NS300 equipped with a 488 nm laser block (Malvern Panalytical, Worcestershire, UK). Samples were diluted 1:5000 (PM) or 1:2500 (TM/IJM) in Tris buffer. Each sample was measured five times for 60 s at ambient temperature, with flow settings at a constant setting of 200, with a camera level of 9 and a screen gain of 6. Processing analysis was conducted using a detection threshold of 4, with the software version NTA 3.4 Build 3.4 003. The span parameter was calculated using $((D90-D10)/D50)$.

4.3.7 *In vitro* Protein Expression of LNPs

HEK-293 cells were seeded at 1.5×10^4 cells/well in either a 96-well white (Fluc) or black (Green Lantern) clear bottom plate in Minimal Essential Media (MEM) (supplemented with 10% FBS, 5% sodium pyruvate, and 5% penicillin streptomycin) and incubated at 37 °C (5% CO₂) for 48 h. Following incubation, cells were treated with LNPs serially diluted to achieve mRNA concentrations of 2–0.25 µg/mL and left to incubate for a further 48 h at 37 °C (5% CO₂). For Fluc-LNPs, to measure results, 100 µL of One-Glo luciferase was added to each well, and luminescence was measured using the Glomax (Promega, Chilworth, UK). For Green Lantern-LNPs, the fluorescence was measured using the POLARstar Omega Plate Reader (BMG LABTECH Ltd., Aylesbury, UK).

4.3.8 Measuring LNP Uptake Using Flow Cytometry

For LNP uptake, cells were plated in a 12-well plate at a concentration of 5×10^5 cells/well and incubated for 48 h at 37 °C (5% CO₂). Then, 24 h pre-analysis, the dead cell control was treated with 0.1% Triton-MEM and 4 h pre-analysis, LNPs were added to the appropriate well at a mRNA concentration of 2 µg/mL. Sample

preparation was carried out at room temperature, spent media was removed from wells and cells were washed with DPBS; TrypLE was added to all wells and cells were allowed to dissociate at 37 °C (5% CO₂) for 2 min. After this, MEM was added to all wells, and samples were spun at 300× g for 5 min until a cell pellet was observed. The supernatant was removed, and the cell pellet was resuspended in DPBS, and where appropriate, 2 drops of propidium iodide were added. Samples remained at room temperature for 15 min before being transferred onto ice until analysis was complete. Flow cytometry measurements were done using the Attune NxT Flow Cytometer (Thermo Fisher) using the BL2 laser to monitor PI and the RL1 laser to monitor Cy5 fluorescence.

Flow cytometry analysis was gated based off of three control groups, unstained live cells (Figure 4-11), stain live cells (Figure 4-12) and stained dead cells (Figure 4-13), once gating was appropriate for all three controls, analysis of samples was undertaken.

4.3.9 *In vivo* Protein Expression Studies

For comparing mixers, 14-week-old female BALB/c mice were intramuscularly injected with 50 µL/hind leg of LNPs, equating to 2 µg total Fluc mRNA. Then, 0.5 h post-injection, mice were subcutaneously injected with 100 µL of Vivo-Glo Luciferin, and after 10 min, bioluminescence signals were captured using an IVIS Spectrum imaging system (Revvity, Inc., Waltham, MA, USA). Bioluminescence signals were further captured at 0.5-, 6- and 24-h post-injection. For comparing formulations, 8-week-old male BALB/c mice were intramuscularly injected with 50 µL/hind leg of LNPs, equating to 2 µg total Fluc mRNA. Bioluminescence signals were captured as mentioned above, at 6- and 24-h post-injection. Bioluminescence signals were quantified using the Living Image 4.0 Software.

4.3.10 Cell Free Protein Expression (CFPE)

A Rabbit Reticulocyte Lysate (RRL) system was used to analyze CFPE. Briefly, Fluc mRNA was extracted from LNPs by mixing 200 µL of sample with 750 µL ethanol

and 25 μ L sodium acetate at 3 M pH 5, before being centrifuged at 14,000 rpm for 20 min. Ethanol precipitation was carried out twice, and the resulting mRNA pellet was resuspended in 35 μ L of RNase-free water. Extracted mRNA was then used to complete reaction mixtures consisting of 35 μ L rabbit reticulocyte lysate, 0.5 μ L of both amino acid mixtures minus leucine or methionine, 1 μ L RNasin[®] Ribonuclease Inhibitor, 2 μ L of extracted mRNA, control mRNA (positive) or no mRNA (negative), and made up to a final volume of 50 μ L with ultrapure water. The reaction mixtures were incubated at 30 °C for 1.5 h, and the reaction was terminated by placing them on ice. To measure protein expression, reaction mixtures were mixed with an equal volume of ONEGlo luciferase and luminescence was measured on the Glomax (Promega).

4.3.11 High Performance Liquid Chromatography – Evaporated Light Scattering Dectector (HPLC-ELSD)

Lipid recovery was measured using the Shimadzu HPLC-ELSD III system. LNPs were injected at a volume of 10 μ L at a flow rate of 0.5 mL/min, and lipids were separated using a Waters Column XBridge[®] C18 Column (internal diameter 3 mm, column length 150 mm, pore size 130 Å, and 3.5 μ m particle size) with a detection temperature of 50 °C and a column oven temperature of 45 °C. Mobile phase A consisted of 0.01 M Triethylamine acetate and water, while mobile phase B consisted of 0.01 M Triethylamine acetate and ethanol. The run time was 15 min with a binary gradient, with mobile phase B increasing from 70 to 100% from 0 to 7 min with a drop back to 70/30% B/A gradient at 12.1 min. Samples were diluted 1:5 (v/v) with mobile phase B before analysis. Concentration curves were developed for each lipid, and all analysis was done using the Shimadzu post-run software v5.124 SP1.

4.3.12 Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4) Multiplexed with Multiangle Light Scattering, Dynamic Light Scattering, and UV Detection

The asymmetric flow field-flow fractionation module (AF2000, Postnova Analytics, Malvern, UK) was hyphenated with an inline multidetector (MD), multiangle light scattering (MALS-PN3621, Postnova Analytics), UV detector (PN3242, 260 nm, 0.5 sensitivity—Postnova Analytics), and DLS Zetasizer Nano ZS System (Malvern Panalytical, Worcestershire, UK). A frit-inlet channel (300 mm × 60 mm × 40 mm) used a 350 µm spacer, 10 kDa MWCO amphiphilic regenerated cellulose membrane and 10 mM pH 7.5 Tris as the eluent liquid for separation at 25 °C. The FI-AF4-MD methodology was used from Davidson et al. [201] with a cross-flow (XF) initial hold of 15 min at 0.75 mL/min, followed by a 60 min exponential XF decay from 0.75 mL/min to 0 mL/min and a hold of 0 mL/min for 5 min; throughout separation, a detector flow (DF) of 0.3 mL/min was used.

PM, TM, and IJM LNP samples were diluted to 0.5 mg/mL lipid concentration in eluent liquid prior to analysis. A 20 µL sample was injected using a 100 µL injection loop. The sample recovery, radius of gyration (R_g) from the MALS detector, hydrodynamic radius (R_h) from the inline DLS, and shape factor were calculated as per Abdulrahman et al. [202]. The collected MALS data were analyzed using the AF2000 software v2.2.01 (Postnova Analytics, Germany), which uses a spherical fit model across 52°–124° angles.

4.3.13 High-Throughput Formulation of LNPs Using Automated Platform

The aqueous phase consisted of Green Lantern mRNA diluted in 5 mM pH 4.5 citrate buffer. The mRNA concentration was kept at 0.05 mg/mL in all formulations. The lipid phase again consisted of DSPC, cholesterol, and DMG-PEG2000 and SM-102, D-Lin-MC3-DMA, DODAP, or DOTAP at a molar ratio of

10:38.5:50:1.5% dissolved in ethanol. The lipids and mRNA solutions were mixed in a robotic liquid handling system (JANUS G3 automated workstation). A robotic liquid handling system prepared the lipid mixture and mRNA dilutions for the high-throughput process. The mixing volume ratio of the aqueous/lipids in ethanol was maintained at 3:1, and the pipetting speed was set at 600 $\mu\text{L/s}$. LNP formulations were diluted 10-fold in Tris sucrose buffer to achieve a final concentration of 3.75 $\mu\text{g/mL}$ mRNA.

4.3.14 Physicochemical Characterisation of LNPs by Automation

Critical quality attributes (CQAs) of LNPs were monitored using Dynamic Light Scattering (DLS). The z-average diameter (nm) and polydispersity index of LNPs were measured in a 96-well plate using a DynaPro plate reader II (Wyatt Technology UK Ltd., Wilmslow, UK). LNPs were diluted 10-fold in Tris buffer using a CyBio FeliX liquid handler (Analytik Jena UK Ltd., London, UK) for size measurement. Solvent parameters for sucrose 2% were applied for analysis, including a viscosity of 1.055 cP and refractive index of 1.336 (25 °C).

The payload encapsulation efficiency (EE%) was quantified using the Quanti-iT™ RiboGreen™ RNA Assay Kit (Invitrogen™) following the manufacturer's instructions and adapted to be performed on a CyBio FeliX liquid handler (Analytik Jena), integrated with a PreciseFlex 750 robotic arm, STX44 incubator (LiCONiC, Montabaur, Germany), labware hotel, barcode reader, and Synergy H1 microplate reader (BioTek, Winooski, VT, USA). A high (0–1000 ng) standard curve was prepared using the encapsulated payload to quantify RNA from Triton-treated and non-Triton-treated wells. Briefly, samples were diluted to a concentration of 1 $\mu\text{g/mL}$ mRNA and mixed with 50 μL of either 2% Triton-TE or 1 $\times$ TE buffer before incubation at 37 °C for 15 min. Following incubation, a 200 $\times$ RiboGreen solution was added to all wells for a final 1:1 dilution. Fluorescence was then measured at an excitation of 485 nm and an emission of 535 nm.

4.3.15 High Throughput *In vitro* Assay

A high-throughput cell-based *in vitro* assay for interrogation of LNP efficacy was developed by the IDDC consortium to support formulation screening. LNPs encapsulating mGreenLantern mRNA were dosed using an Echo liquid handler at 1 ng, 10 ng, or 30 ng mRNA per well in a 384-well plate (Greiner μ CLEAR®, Nürtingen, Germany). HeLa cells, which were previously modified by lentiviral transduction to express nuclear-localized mKate2, were seeded on top of LNPs at 2×10^3 cells per well in FluoroBrite DMEM (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA), GlutaMAX™ Supplement (Gibco) and 1% penicillin–streptomycin (Sigma), and incubated at 37 °C (5% CO₂) for 42 h. Following incubation, cells were fixed with 4% paraformaldehyde and imaged using an Opera Phenix high-content screening system equipped with a 10 × 0.3 NA air objective and 488 nm and 561 nm laser lines. Images were analyzed in Harmony. Automated segmentation pipelines were constructed to identify nuclei and cytosolic mGreenLantern expression with single-cell resolution. The cell number was defined as the number of detected nuclei per well, and transfection efficiency as the proportion of these cells positive for mGreenLantern expression. Data were normalized to internal controls before plotting in Microsoft Excel (Version 16.84, Microsoft Corporation, Redmond, WA, USA).

4.3.16 Cryogenic Transmission Electron Microscopy

Fluc-LNP samples were prepared as normal and then concentrated to 0.08 mg/mL mRNA. Glow discharge was carried out on Lacey carbon film, 300 mesh copper grids (Agar Scientific, Rotherham, UK) for 30 s at 30 mA using the Emitech K100X (Quorum Technologies Ltd., Laughton, UK). A total of 3 μ L of the LNP samples were blotted on either Whatman 597 paper using the Thermo Fisher Vitroblot Mk IV (25 °C, 95% humidity, blot time 3 s, and blot force 13 or 12) or Whatmann 1 paper using the Leica EM GP2 plunge-freezer before being plunge

frozen in liquid ethane. Samples were cooled in liquid nitrogen and held in a Gatan Elsa cryo-specimen holder until being transferred to the JEOL F2-Cryo (200 kV) with Direct Electron DE-20 camera for image capture. The acquisition software was SerialEM version 4.1, with images being processed on Relion 5.0.0 and Image J.JS v0.5.8.

4.3.17 Ethics Statement

Animal experiments and experimental procedures were carried out in accordance with UK Home Office regulations and the University of Strathclyde Animal Welfare and Ethical Review Board regulations under project license number PPL PP1650440. BALB/c mice were bred and maintained in the Biological Procedures Unit at the University of Strathclyde, Glasgow, and experiment design and reporting adhered to the ARRIVE guidelines.

4.3.18 Statistical Analysis

All results represent the mean $\pm$ standard deviation of three independent replicates. Statistical significance was determined using an ANOVA with Tukey's post hoc test using Minitab software (v19.2). For *in vivo* studies, 2–3 mice were used for each condition to give 4–6 regions of interest for the analysis of the injection site and 2–3 regions of interest for the analysis of livers, with results representing the mean $\pm$ standard error of the mean (SEM). No data points were excluded.

4.4 Results

4.4.1 Physicochemical Properties of LNPs Are Dependant on the Payload Encapsulated

A vital component of LNP-based formulations is the nucleic acid payload, with particular emphasis on how variations in the payload can affect the CQAs of the resulting LNPs. Throughout this study, four nucleic acid payloads were used: poly A, Firefly Luciferase mRNA (Fluc), Green Lantern mRNA (GrL) and Cy5-labelled

Firefly Luciferase mRNA (Cy5-Fluc). The results in Figure 4-2 and Table 4-1 show that switching between these payloads led to variations in CQAs, highlighting the impact of payload selection on LNP characteristics.

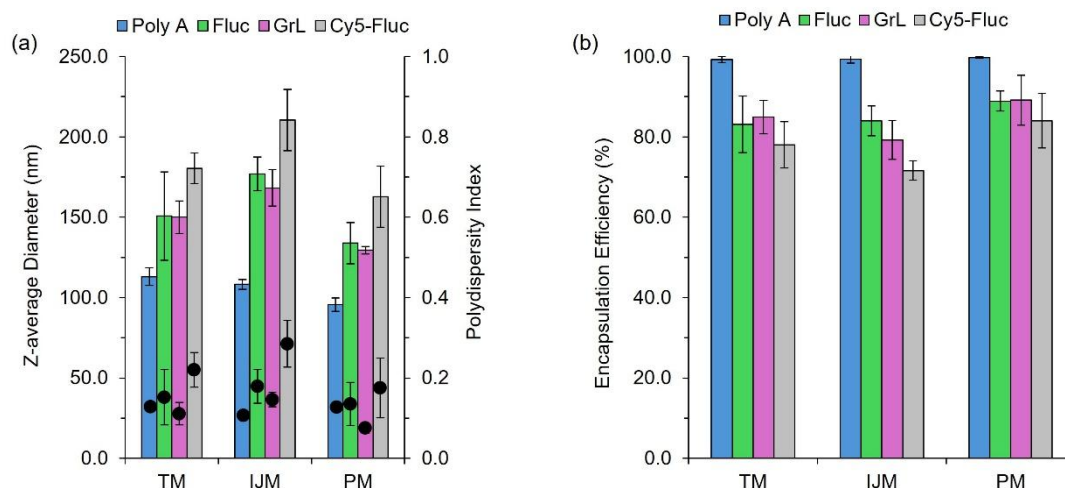


Figure 4-2 The effect of payload on LNP CQAs. LNPs composed of DSPC:chol:SM-102:DMG-PEG2000 (10:38.5:50:1.5%) encapsulating varying nucleic acid payloads. (a) demonstrates the Z-average diameter (nm) (bars) and polydispersity index (circles). (b) show the encapsulation efficiency (%). Results represent the mean $\pm$ standard deviation (SD) ($n = 3-4$). TM: T-junction mixer, IJM: confined impingement jet mixer, PM: pipette mixing, Fluc: Firefly Luciferase mRNA, GrL: Green Lantern mRNA.

Table 4-1 The zeta potential (mV) of LNPs encapsulating various nucleic acid payloads. Results are representation of mean $\pm$ standard deviation ($n = 3-4$).

Mixer	Poly A	Fluc	GrL	Cy5-Fluc
TM	1.6 $\pm$ 1.2	0 $\pm$ 2.2	2.4 $\pm$ 1.9	-0.2 $\pm$ 0.6
IJM	0.7 $\pm$ 1.3	-0.6 $\pm$ 2.2	2.9 $\pm$ 1.2	-1.3 $\pm$ 2.0
PM	2.3 $\pm$ 1.3	1.6 $\pm$ 3.1	2.5 $\pm$ 2.1	1.3 $\pm$ 1.3

Figure 4-2a highlights that, regardless of the manufacturing method used, particle size and polydispersity (PDI) differences can be seen depending on the nucleic acid payload. For all mixers, poly A-LNPs were significantly ($p < 0.05$) smaller in size compared to the other formulations, while Cy5-Fluc-LNPs resulted in LNPs significantly larger ($p < 0.05$) compared to the other LNPs. In contrast, varying between mRNA constructs (Fluc-LNPs vs. GrL-LNPs) had no significant effect. Cy5-Fluc-LNPs also exhibited a significant increase in PDI ($p < 0.05$) when manufactured using the TM or IJM methods. For Fluc-, GrL- and Cy5-

Fluc-LNPs, manufacturing on the IJM also resulted in significantly ($p < 0.05$) larger particle sizes. For LNPs manufactured on either the TM or IJM, a significant ($p < 0.05$) decrease in encapsulation efficiency was seen for LNPs encapsulating mRNA compared to LNPs encapsulating poly A (Figure 4-2b). The results in Table 4-1 indicate that all mixing methods produced LNPs with zeta potential values near neutral across all nucleic acid payloads, with no significant differences observed between mixers.

4.4.2 Low-Cost Microfluidic Mixers Can Produce LNPs with Sufficient *In vitro* Protein Expression

LNP *in vitro* efficiency was determined using HEK-293 cell models with either a Firefly Luciferase (Fluc) or a Green Fluorescent protein (GrL) mRNA construct. Figure 4-3 highlights that all LNPs had similar expression, viability, and uptake regardless of the microfluidic mixer used.

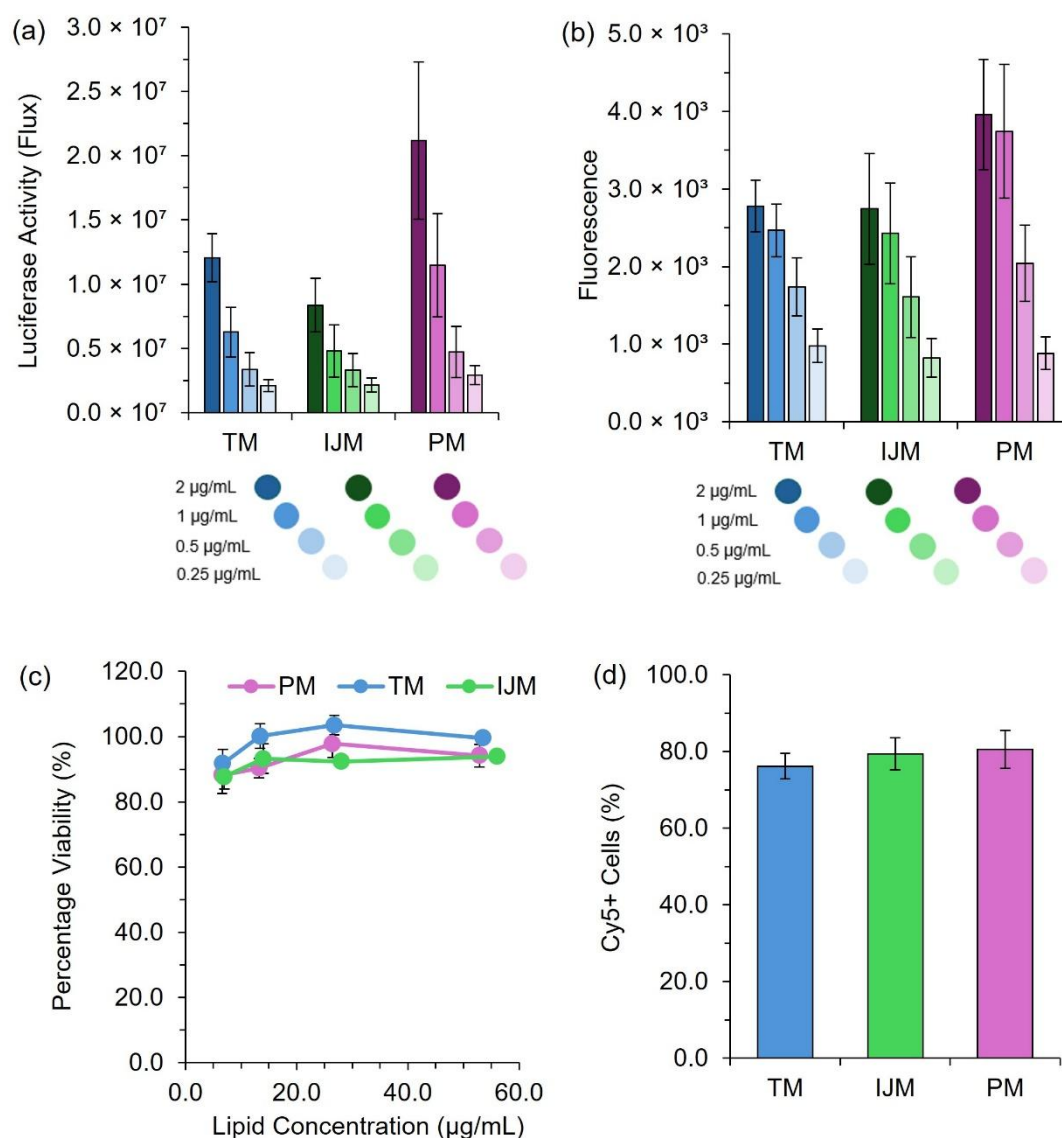


Figure 4-3 *In vitro* performance of LNPs. HEK-292 cells were treated with either Fluc-LNPs or GrL-LNPs at mRNA concentrations of 2 µg/mL to 0.25 µg/mL, and luminescence signals (a) or fluorescence signals (b) were monitored 48 h after transfection as a direct measure of protein expression. (c) demonstrates the percentage viability of HEK-293 cells 48 h after transfection with GrL-LNPs, while (d) demonstrates the percentage of Cy5+ cells 4 h after transfection with Cy5-Fluc-LNPs measured using flow cytometry. All results are representative of the mean ± standard error of the mean (SEM) (n = 3).

Regardless of the mRNA construct used, LNPs manufactured using TM, IJM, and PM all promoted *in vitro* either Firefly Luciferase or green fluorescent protein expression (Figure 4-3a,b), with no significant difference observed between the formulations. No toxicity issues were noted across all treatment concentrations and mixing methods tested (Figure 4-3c). To assess cellular uptake, LNPs encapsulating Cy5-Fluc were manufactured and used as a direct uptake

indicator. Figure 4-3d shows that four hours post-transfection, all LNP formulations exhibited high uptake in HEK-293 cells, with ~80% Cy5+ cells observed across all mixers, confirming efficient cellular internalization.

4.4.3 Determining Lipid and mRNA Integrity of LNPs

Post-manufacture monitoring of LNPs is essential for ensuring the stability and efficacy of LNP-based formulations. While CQAs such as particle size play a crucial role in formulation performance, assessing the integrity of the lipid and payload components is equally important. To achieve this, in addition to testing *in vitro* and *in vivo* efficacy, a cell-free protein expression system was employed to evaluate mRNA expression levels following extraction from LNPs, while HPLC-ELSD analysis was used to assess lipid integrity and ensure high lipid recovery (Figure 4-4).

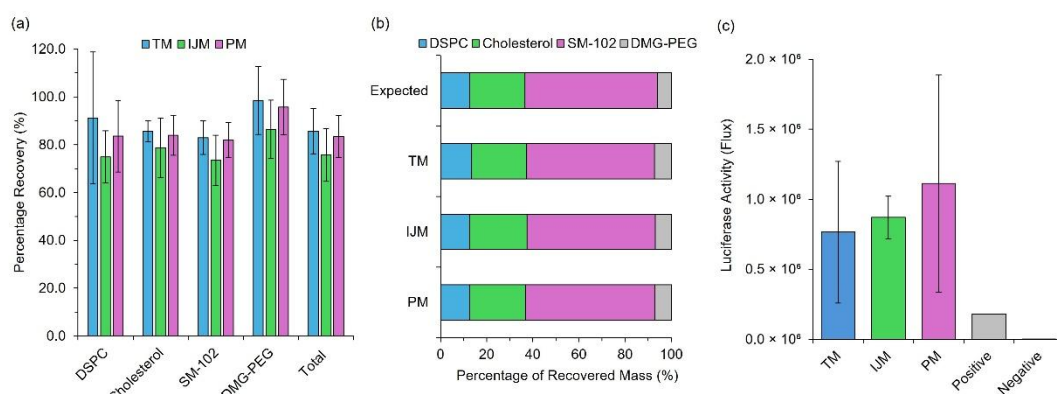


Figure 4-4 Post-manufacture integrity monitoring of LNPs. (a) represents the individual and total lipid recovery measured using Shimadzu HPLC-ELSD III of GrL-LNPs, while (b) represents the respective recovery in terms of the mass ratio of lipids. (c) represents the luciferase activity (flux) as a direct measure of protein expression produced from mRNA extracted from Fluc-LNPs and tested in a Rabbit Reticulocyte Lysate cell-free protein expression system. Results represent the mean $\pm$ standard deviation (SD) ($n = 3$).

Regardless of the manufacturing method used for LNPs, all formulations had total recoveries greater than 75%, with individual lipid recoveries ranging between 73 and 100% (Figure 4a), and all formulations resulted in mass ratios similar to the expected mass ratio—varying with <2% error across lipids (Figure 4-4b). This ensures that, although some product loss is inevitable, it occurs at the same rate across all lipid components. Unequal loss, particularly of critical

components such as the ionizable lipid, could significantly compromise LNP functionality. Finally, extracted mRNA from the formulations was then used to treat CFPE systems at a concentration of 2 $\mu\text{g/mL}$, and this showed similar results to *in vitro* testing with no significant differences in expression levels (Figure 4-4c). These results confirm that low-cost microfluidic mixers do not compromise the integrity or functionality of LNPs.

4.4.4 Pipette-Mixed LNPs Exhibit Comparable *In vivo* Protein Expression to Low-Cost Microfluidic Mixers

For *in vivo* protein expression, BALB/c mice were treated with LNPs encapsulating Fluc mRNA and imaged on the IVIS Spectrum (Figure 4-5). Due to the similar CQAs between TM and PM samples, both LNPs were included in the *in vivo* studies to determine whether manual mixing had any significant effect on the protein expression of the LNPs.

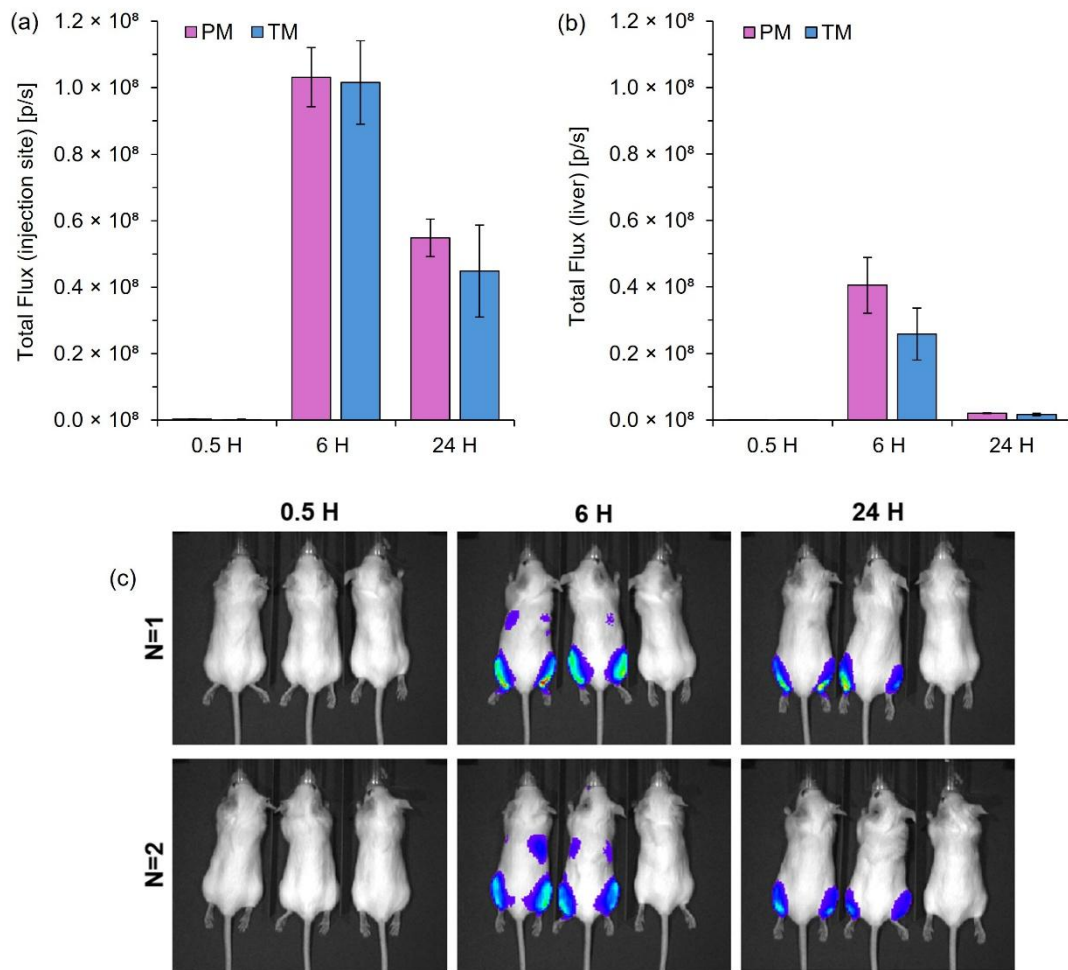


Figure 4-5 *In vivo* protein expression following IM injection of mRNA-LNPs prepared by pipette mixing (PM) or T-junction mixing (TM). Quantification of total flux at the injection site (a) and liver region (b) at 0.5, 6, and 24 h post-injection. (c) Representative images of the BALB/c mice (mouse order from left to right: PM, TM, and control mouse). The colour scale represents the luminescence intensity in radiance (p/s/cm²/sr), ranging from 0.5×10^7 (blue) to 2.0×10^7 (red); the minimum and maximum values recorded were 7.0×10^5 and 1.8×10^7 p/s/cm²/sr, respectively. Bioluminescence signals were quantified using the Living Image 4.0 software, with regions of interest defined at the injection point (a) and liver (b). Results are expressed as the mean $\pm$ standard error of the mean (SEM) ($n = 4$ for leg; $n = 2$ for liver).

Figure 4-5 highlights that LNPs made via a manual pipette mixing process (PM) with no control over speed could produce protein expression levels similar to that of an automated controlled T-junction mixer (TM) with no statistically significant differences. Protein expression levels were highest 6 h post-injection, with sustained expression evident 24 h post-injection (Figure 4-5a). Moderate levels of expression can also be seen in the liver 6 h post-injection, but were minimal at

the 24 h time point (b). The results in Figure 4-5 highlight PM as an easy, quick, and cheap alternative to microfluidic mixing in bench-scale research.

4.4.5 Orthogonal Analysis of LNPs

While PM, TM, and IJM showed no statistically significant variations *in vitro*, and PM and TM demonstrated comparable *in vivo* efficacy, further detailed orthogonal analyses were conducted to investigate physicochemical differences between LNPs produced using different mixers. To achieve this, three complementary techniques were employed. Dynamic Light Scattering (DLS) provided a rapid average measurement of a population's physicochemical properties, including size and polydispersity, as shown in Table 4-2. Nanoparticle Tracking Analysis (NTA) provides a more detailed assessment of individual particle sizes and concentrations, reporting results as number-based rather than intensity-based values. This enables measurement of the size and polydispersity of the nanoparticle population without larger particles dominating the intensity signal and skewing particle size average; these results are presented in Table 4-3 and the distribution plots are shown in Figure 4-6. Asymmetric flow field-flow fractionation (AF4) with inline detectors offers enhanced resolution by separating particles based on the Stokes–Einstein equation. This technique allows for the analysis of additional characteristics, such as the radius of gyration (R_g). The R_g reflects the distribution of the mass within a particle and, when coupled with the hydrodynamic radius (R_h), can give insights into the shape factor of the LNPs, indicating whether they have a spherical shape. These results are detailed in Table 4-4 and Figure 4-7. By employing these orthogonal techniques, key physicochemical properties, including size, polydispersity, distribution profiles, R_g , particle concentration, and shape factor, were examined to better understand the influence of mixer selection on LNP characteristics.

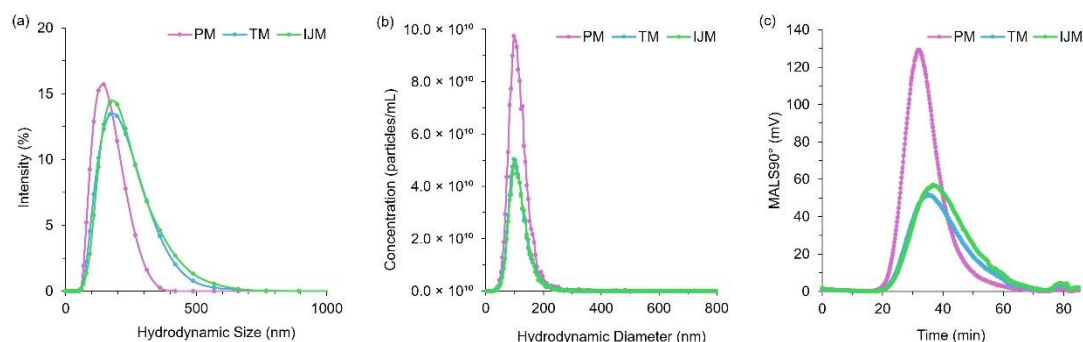


Figure 4-6 Distribution profiles for PM, TM, and IJM Fluc-LNPs: (a) hydrodynamic size by DLS (intensity distribution), (b) hydrodynamic size by NTA (particle concentration, particles/mL) and (c) MALS 90° signal by AF4-MALS. Data represent averages from two independent runs per method, comprising ten measurements (NTA), six measurements (DLS), and six (PM/TM) or five (IJM) injections (MALS).

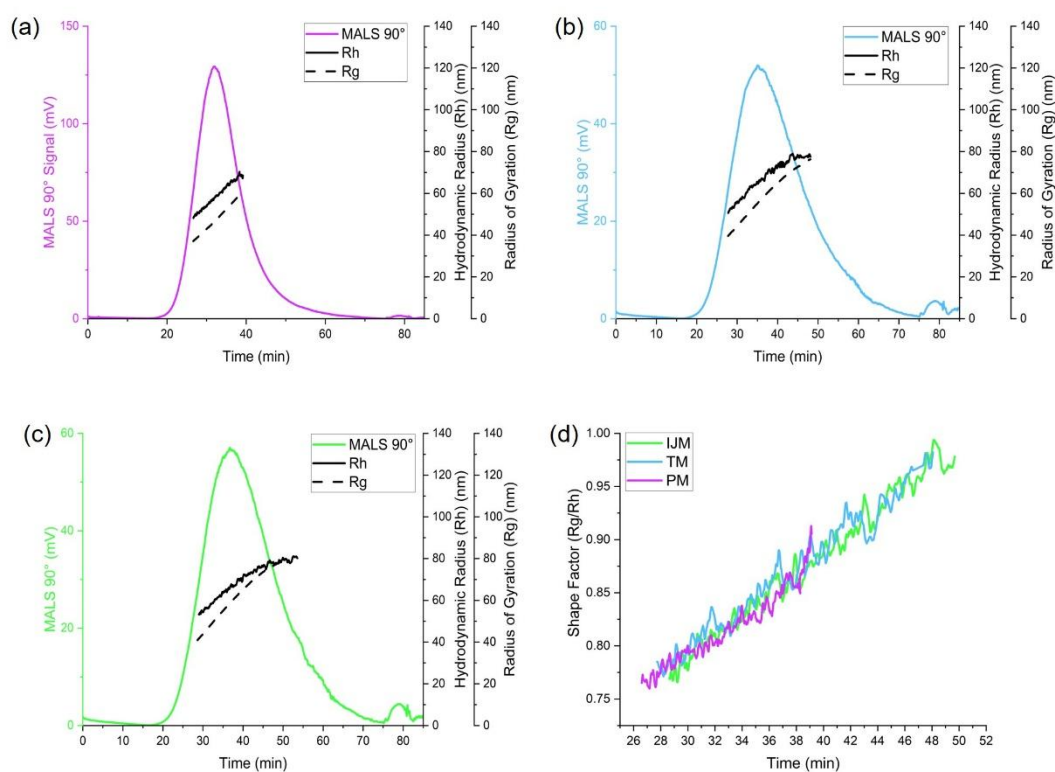


Figure 4-7 Analysis of Fluc-LNPs manufactured on either the PM (a), TM (b), or IJM (c), represented by the scattering intensity at the MALS 90° scattering angle (colored lines), hydrodynamic radius (Rh) (black line), and radius of gyration (Rg) (dotted black line). (d) represents the shape factor over time for all three manufacture methods. Results are representative of the mean $\pm$ standard deviation (SD) of two independent repeats consisting of six (PM/TM) or five (IJM) injections.

Table 4-2 Summary of physicochemical data collected from Zetasizer Ultra (DLS).

Dynamic Light Scattering (DLS)		
Mixers	Z-average Diameter (nm)	PDI
TM	170 ± 6	0.199 ± 0.018
IJM	186 ± 16	0.228 ± 0.035
PM	136 ± 6	0.112 ± 0.018

Table 4-3 Summary of Physicochemical data collected from Nanosight Pro (NTA).

Nanoparticle Tracking Analysis (NTA)							
Mixers	Mean (nm)	Mode (nm)	D10 (nm)	D50 (nm)	D90 (nm)	Span	Concentration (particles/mL)
TM	113 ± 5	102 ± 6	76 ± 4	107 ± 6	157 ± 9	0.75	7.06 x 10 ¹¹
IJM	115 ± 5	100 ± 9	78 ± 3	110 ± 5	158 ± 6	0.73	6.87 x 10 ¹¹
PM	113 ± 8	102 ± 10	78 ± 7	108 ± 8	154 ± 11	0.71	1.35 x 10 ¹²

Table 4-4 Summary of physicochemical data collected from AF4-MALS.

AF4-MALS							
Mixers	Et-(min)	Rec (%)	Rg Mode (nm)	Rg-D10 (nm)	Rg-D50 (nm)	Rg-D90 (NM)	Rg-Span
TM	38 ± 2	79 ± 9	60 ± 5	44	59	72	0.48
IJM	39 ± 2	90 ± 22	63 ± 2	46	62	77	0.50
PM	33 ± 0.1	96 ± 8	47 ± 0.4	39	47	57	0.37

The use of the orthogonal techniques highlights the range of size and heterogeneity information that can be collated for LNPs, and while all the methods provide different readouts, the pattern of PM producing smaller, more monodisperse particles compared to TM or IJM remained the same (Table 4-2, Table 4-3 and Table 4-4). Overall, it was shown that the AF4 method was successful across all mixers, with formulations reaching a recovery of ~80% for TM and ~90% for both PM and IJM, which aligned with the ISO standard [160] (Table 4-4). PM demonstrated the largest MALS 90° signal with minimal sub-populations, indicating enhanced diffusion and separation within the channel. This could be attributed to the increased particle concentration, as confirmed by the NTA. Results from the NTA showed less variation in particle size across the mixers when compared to DLS due to NTA generating a number-based measurement rather than an intensity-weight distribution. Regardless of this, PM LNPs consistently showed narrower particle sizes and more monodisperse

populations across DLS, NTA, and AF4 (Figure 4-6). On the other hand, TM and IJM produced wider MALS 90° peaks with an increased number of shouldering peaks, indicating a higher presence of sub-populations (Figure 4-7a,b). TM and IJM also exhibited wash-off of aggregates when the crossflow reached 0 mL/min, eluting as a small peak after 75 min, which is during the membrane wash, indicating wash-off of unseparated larger aggregates (Figure 4-7b,c). Measurement of the ratio between R_g and R_h values also allowed for the shape factor of the LNPs to be analyzed, where it was seen that all LNPs exhibited similar spherical shape factors of ~ 0.775 . Due to the shorter and wider area of the TM and IJM peaks, these LNPs have an increased FWHM, leading to increased shape factors as time increases, indicating possible oval or conjoined string shapes for LNPs (Figure 4-7d). Finally, the R_g -span from AF4 and span calculated from NTA agrees with the PDI measured by DLS, indicating that regardless of the orthogonal technique employed, larger sizes and increased heterogeneity were noted when LNPs were manufactured using TM or IJM.

4.4.6 Cryo-TEM Images of LNPs

Cryo-TEM was used to gain further insight into the structure of LNPs prepared by the microfluidic and manual mixing procedures. Figure 4-8 presents representative cryo-TEM images of LNPs produced via PM, TM, and IJM. All mixing methods produced particles with distinguishable blebs. Across all formulations, particles were observed to contain no blebs, single blebs, or multiple blebs, with no apparent differences in the frequency or distribution of these features between mixing methods.

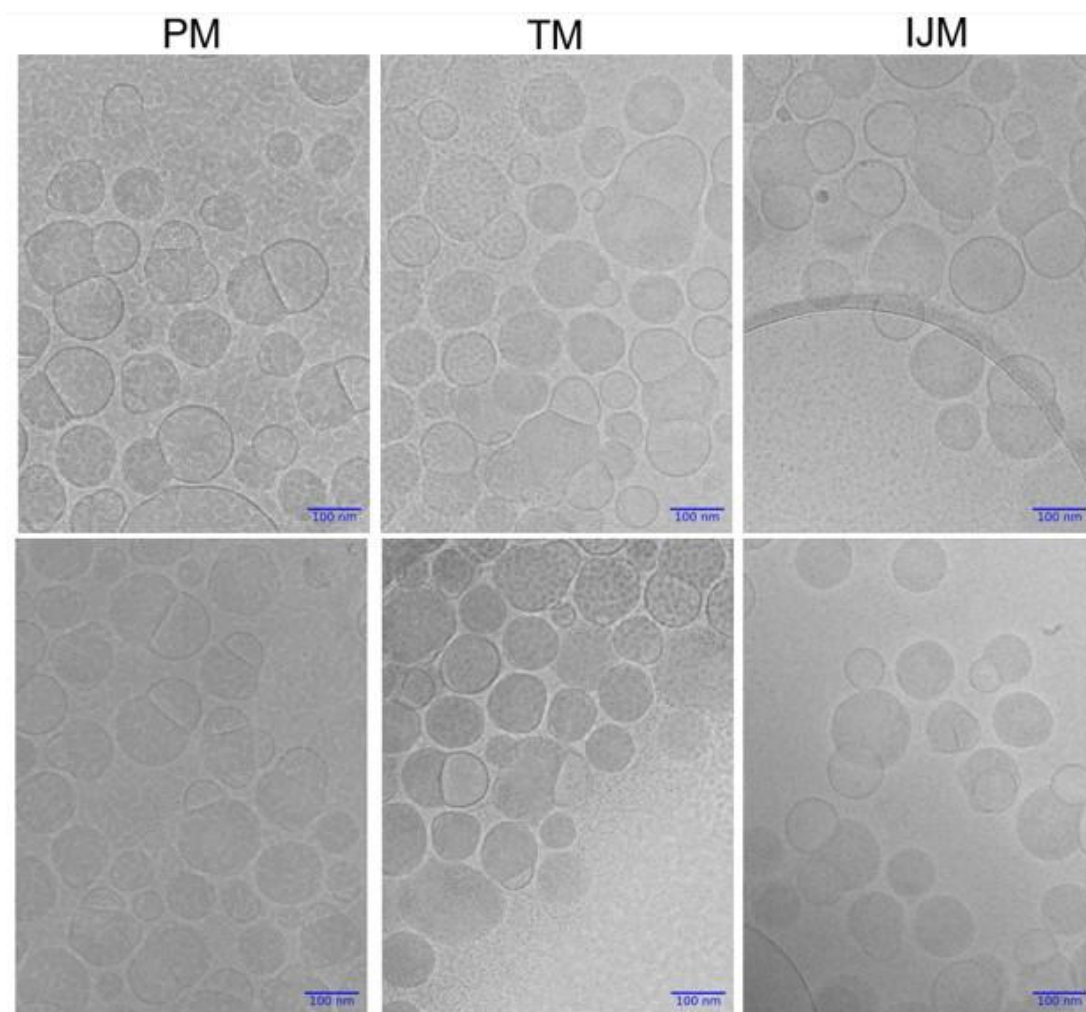


Figure 4-8 Cryogenic Transmission Electron Microscopy (cryo-TEM) images of Fluc-LNPs manufactured using pipette mixing (PM), T-junction mixing (TM), or impingement jet mixing (IJM). Two images are available for each condition with magnification set at 30,000 $\times$ with a 100 nm scale bar for all images. Images were captured on JEOL F2-Cryo (200 kV), while image processing was completed using Relion 5.0.0 and Image J.

4.4.7 Exploiting Pipette Mixing as a Cost-Effective High-Throughput Screening Platform

Given that our results demonstrate that pipette mixing can produce small monodisperse LNPs and promote mRNA expression both *in vitro* and *in vivo*, we next sought to determine if this approach could effectively discriminate among diverse LNP formulations when used for rapid screening. This would enable its integration into an automated platform for high-throughput formulation development. By systematically assessing key metrics (such as particle size,

polydispersity index, and encapsulation efficiency) across multiple pipette-mixed LNP formulations, we aimed to confirm that pipette mixing can provide a practical approach for screening LNP formulations.

To validate the pipette-mixing approach, we prepared four widely reported LNP formulations, SM102, DLin, DOTAP, and DODAP, each known to exhibit distinct *in vivo* expression profiles [77]. These formulations were produced in a high-throughput manner using pipette mixing, and their physicochemical characteristics and efficacy were subsequently evaluated *in vitro* (Figure 4-9). To finalize this section, we also produced these four formulations using the manual pipette mixing (PM) method to establish key metric differences and evaluate the *in vivo* efficacy (Figure 4-10).

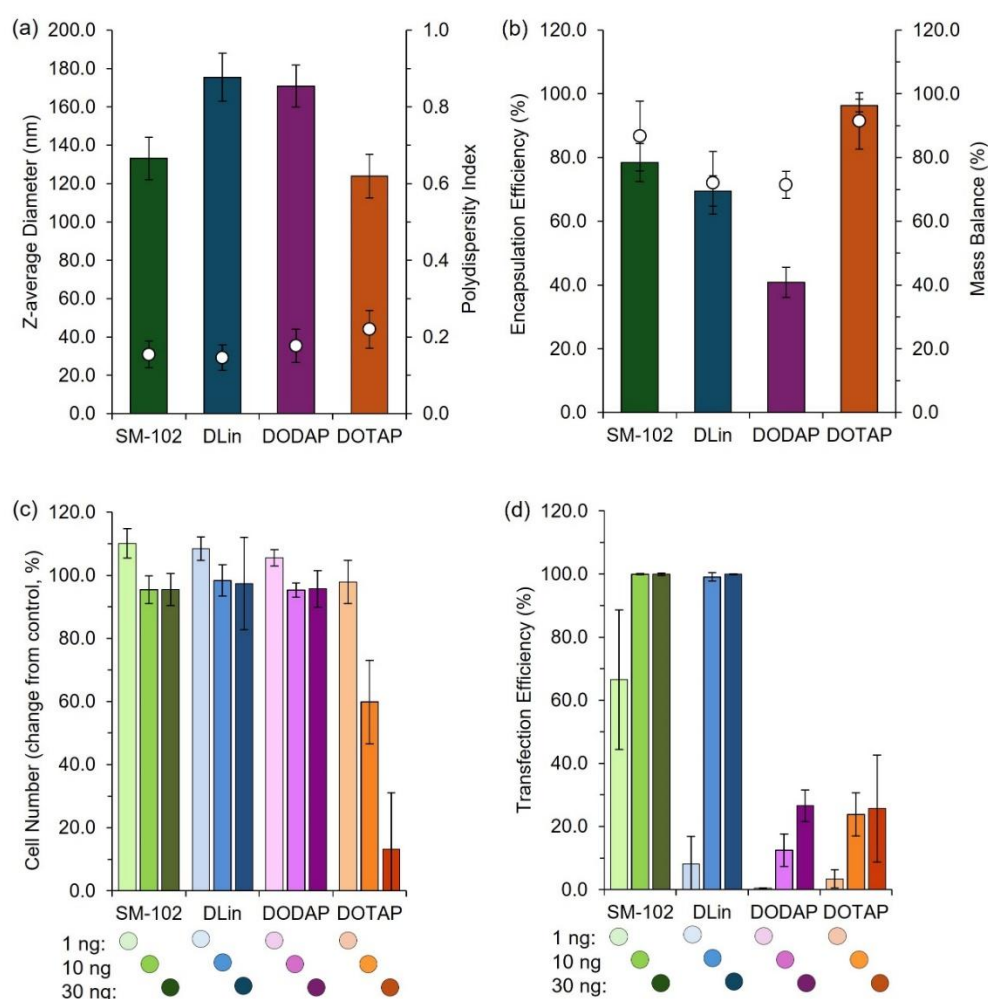


Figure 4-9 High-throughput LNP formulation and characterization platform. LNPs were formulated using SM-102, DLin-MC3-DMA (DLin), DODAP, or DOTAP as the ionizable or cationic lipid components and prepared using a high-throughput (HT) pipette mixing protocol (see Section 2.15 for details). **(a)** Z-average diameter (bars) and PDI (circles) of LNPs prepared via HT, **(b)** encapsulation and mass balance of LNPs prepared via HT. The LNP efficacy was evaluated by quantifying the change in cell number **(c)** and transfection efficiency **(d)** in a high-throughput cell-based transfection assay. LNPs containing mGreenLantern mRNA were reverse-transfected into a HeLa cell line expressing nuclear-mKate2 at 1 ng, 10 ng, or 30 ng mRNA dose per well on a 384-well plate. Cells were imaged after 42 h incubation using an Opera Phenix high-content screening system and quantified using automated analysis in Harmony. Data are normalized to internal controls. Bars show the mean, and whiskers depict the $\pm$ standard deviation.

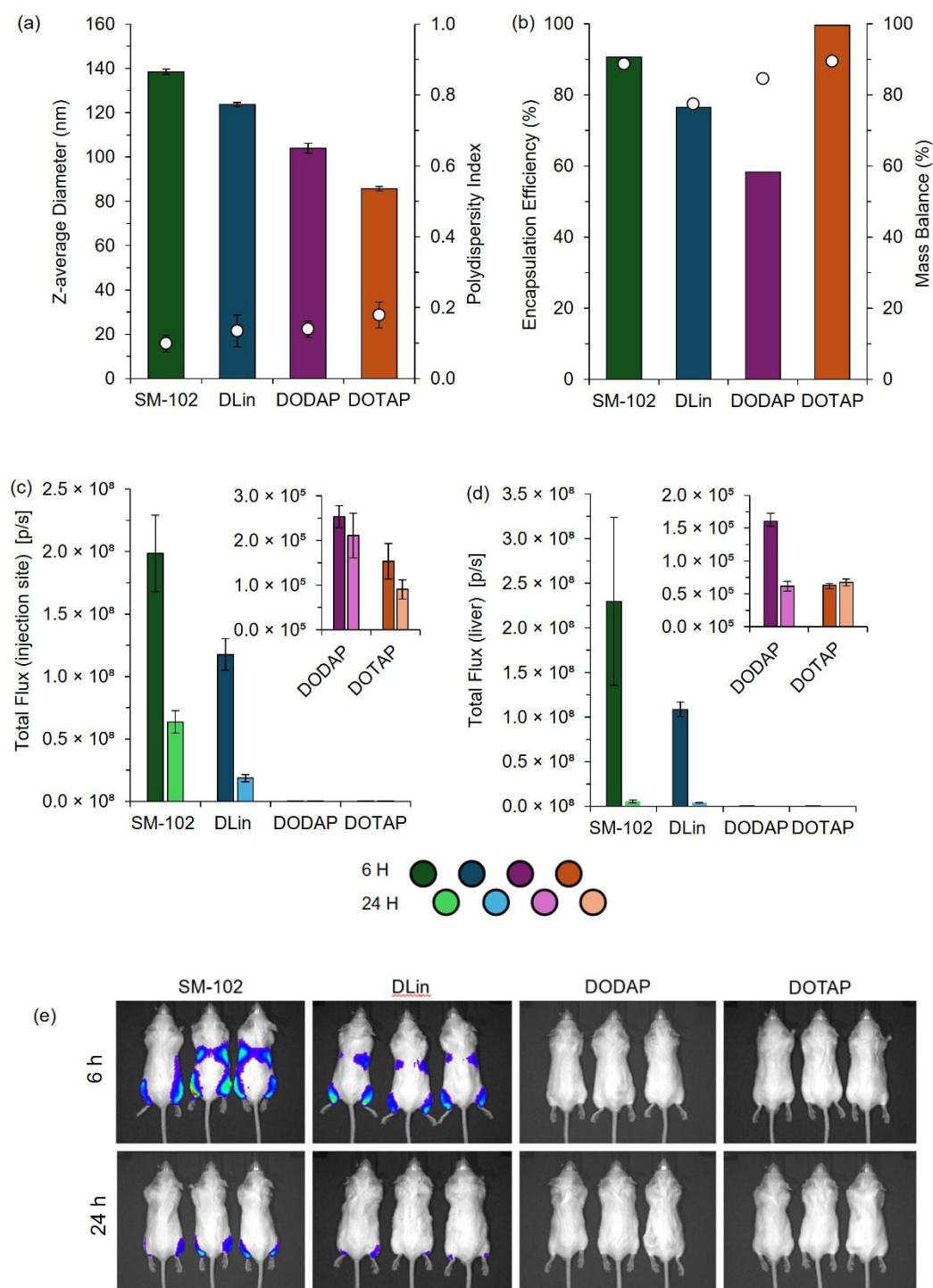


Figure 4-10 Physicochemical characterization and in vivo performance of mRNA-loaded LNPs prepared by manual pipette mixing. LNPs were formulated using SM-102, DLin-MC3-DMA (DLin), DODAP, or DOTAP as the ionizable or cationic lipid components. **(a)** Particle size represented by the z-average diameter (bars) and polydispersity index (circles), determined by DLS. **(b)** Encapsulation efficiency (bars) and mass balance (circles) were quantified using the RiboGreen assay. In vivo total flux at **(c)** the injection site and **(d)** liver region measured at 6 and 24 h following IM administration in BALB/c mice (dose: 1 μ g mRNA per leg). Insets highlight detailed flux data for DODAP and DOTAP formulations. **(e)** Representative bioluminescence images of BALB/c mice at 6 and 24 h post-injection. The color scale indicates the luminescence intensity, ranging from 0.5×10^7 (blue; minimum radiance 2.15×10^6 p/s/cm²/sr) to 2.0×10^7 (red; maximum radiance 2.38×10^7 p/s/cm²/sr). Data are shown as mean $\pm$ SD (size/PDI, $n = 3$ measurements from one batch) or mean $\pm$ SEM (in vivo; injection site $n = 6$, liver $n = 3$).

These results demonstrate distinct differences in efficacy among the formulations tested, and the expression patterns we obtained were consistent with established profiles in the literature [77], confirming the utility of this rapid, cost-effective pipette-based protocol for laboratory studies and high-throughput screening of LNP formulations. This demonstrates that this high-throughput and manual pipette mixing method can effectively discriminate performance across various LNP candidates *in vitro* and in pre-clinical mouse models.

4.5 Discussion

Our research demonstrates that low-cost microfluidic mixers can reliably produce LNPs, highlighting their potential as effective tools for affordable small-scale production and high-throughput screening applications. We evaluated three mixing methods: TM, IJM, and PM. While PM may lack precise control over CPPs and is not scalable, it proved effective for small-scale LNP manufacture. Characterization studies showed that CQAs were influenced more by payload (Figure 4-2) than the mixing method (Figure 4-3, Figure 4-4, Figure 4-5, Figure 4-6, Figure 4-7 and Figure 4-8); for example, poly A yielded smaller LNPs with higher encapsulation efficiencies, whereas mRNA constructs increased particle size. This is likely because poly A is a small, linear molecule compared to mRNA, which can exhibit enhanced secondary structures that may lead to a less tightly packed LNP structure. Cy5-Fluc mRNA also increased the LNP size, possibly due to interactions with the fluorescent tag and lipid components.

We also observed a decrease in encapsulation for LNPs containing Cy5-Fluc (Figure 4-2), possibly due to the Cy5-tag interfering with the RiboGreen assay. Specifically, Cy5 has an excitation/emission spectrum of approximately 650/670 nm, while Ribogreen operates at approximately 500/525 nm; this spectral overlap suggests that Cy5 has the potential to fluorescently quench the RiboGreen signal, thereby affecting the fluorescence readout. To mitigate this effect, Cy5-Fluc mRNA was used during the assay to build the calibration curves. While not

fully quantified in this study, this potential interference should be considered when interpreting encapsulation data involving fluorescently labelled RNA. Fluorescent tags, particularly cyanine dyes, are widely used in LNP and liposomal research and generally do not significantly affect particle characteristics [203]. For example, Ma et al. investigated *in vivo* mRNA delivery using SM-102-LNPs tagged with Cy5 and DiR, reporting sizes around 100 nm [178]. However, their LNPs were produced at a higher flow rate and purified via dialysis. In contrast, we used spin column purification in our studies, introducing a rapid pH change and high centrifugal forces that may contribute to these differences. Schober et al. also examined the impact of payload size in DLin- and ALC-0315-LNPs, finding no significant effect on size [80]. Therefore, the low-cost mixers tested in our studies may be more sensitive to nucleic acid payload, which may also explain differences in encapsulation efficiency.

It is generally recognized that LNP size is a critical factor in determining biodistribution and transfection efficiency, and previous studies have shown that various mixing methods can significantly influence particle size. To investigate this, we used orthogonal characterization techniques to investigate the physicochemical properties of the LNPs produced by different mixers. DLS measurements showed that PM LNPs had smaller hydrodynamic diameters and lower PDI than TM and IJM mixers (Table 4-2 and Figure 4-6). While NTA showed minimal differences in mean particle size across the mixers, PM LNPs displayed a higher particle concentration and narrower size span, indicating a more uniform population (Table 4-3 and Figure 4-6). AF4-MALS analysis supported these findings, with PM LNPs exhibiting lower R_g values and narrower elution profiles, indicating a more compact structure (Table 4-4 and Figure 4-6). Shape factor analysis (Table 4-4 and Figure 4-7) showed all LNPs were generally spherical ($R_g/R_h \sim 0.775$), but TM and IJM particles displayed shape factor that increases over time, suggesting some elongation or fusion events. These differences were not visually evident from cryo-TEM images (Figure 4-8), which showed similar

external morphology and bleb features across all samples. This highlights the importance of employing complementary characterization techniques to fully understand LNP behaviour, as cryo-TEM alone does not easily show population-level differences.

Our findings are generally consistent with the existing literature regarding the impact of mixer choice. For instance, Misra et al. produced SM-102-LNPs using a confined impingement jet system, reporting sizes around 135 nm with high encapsulation efficiency (>95%), which is smaller than the IJM-generated LNPs in this study [204]. However, these LNPs were manufactured under different conditions and included higher PEG content (2 mol%), which has been shown to reduce LNP size [105, 166]. Similarly, Subraveti et al. produced Dlin-MC3-DMA-LNPs using a confined impingement jet mixer, achieving sizes below 100 nm at physiological pH; however, their study also used dialysis purification and had a different lipid composition, which may have contributed to smaller particle sizes [205]. These studies suggest that IJM-generated LNPs can be tuned to smaller sizes by carefully optimizing CPPs, such as the flow rate ratio, lipid composition, and purification method.

Further studies have investigated the impact of size on LNP biodistribution and expression efficiency. Di et al. reported that LNPs ≥ 180 nm exhibited reduced liver expression, while McMillan et al. demonstrated that larger LNPs correlated with significantly decreased *in vivo* expression [105, 206]. Given that TM and PM produced more comparable sizes, they were prioritized for *in vivo* testing, aligning with previous reports that T-mixed LNPs typically range from 120 to 130 nm [89]. Our results showed no significant difference *in vivo* expression between the TM and PM LNPs; however, studies comparing T-junction and chaotic mixers suggest that chaotic mixing methods may improve LNP efficiency. For example, Jürgens et al. found that staggered herringbone mixers (SHMs) outperformed T-mixing for mRNA LNPs [89]. This effect could be attributed to chaotic mixers typically

producing smaller LNPs, which could increase uptake as size-dependent effects on uptake are generally more pronounced at sizes below 100 nm [207].

There are various reports on the performance of pipette-mixed LNPs. For example, Wang et al. found that pipette-mixed MC3-LNPs had larger sizes (~162 nm) and lower mRNA expression than microfluidic-generated LNPs [156]. In contrast, Strelkova et al. demonstrated that manually mixed LNPs could outperform some microfluidic-generated formulations when process parameters were varied [208]. These results, combined with the results we present, confirm that PM is a useful, low-cost option.

To further validate the use of pipette mixing as part of a larger high-throughput pipette mixing system, both high-throughput and manual pipette mixing were employed to generate clinically relevant cationic and ionizable LNPs for *in vitro* and *in vivo* testing. The observed expression profiles aligned with previous reports, with expression increasing from DOTAP > DODAP > DLin > SM-102, consistent with the known efficiency of ionizable lipids (DLin and SM-102) versus cationic lipids (DOTAP and DODAP) in nucleic acid delivery [77]. While PM LNPs exhibited larger sizes, they maintained comparable expression profiles to published data, supporting PM as an effective tool for rapid LNP screening.

4.6 Conclusions

In conclusion, this study demonstrates that low-cost microfluidic mixers can effectively produce LNPs, with pipette mixing (PM) emerging as a viable high-throughput screening tool. While PM, TM, and IJM produced LNPs with similar *in vitro* expression profiles, PM offers a cost-effective and accessible alternative for small-scale research despite lacking precise control over critical process parameters. Orthogonal analysis confirmed no major physicochemical differences besides size variations, and *in vivo* testing showed that PM and TM produced comparable expression profiles, reinforcing the potential of using pipette mixing for predicting LNP potency. A high-throughput pipette mixing

system was successfully validated, demonstrating that PM can be used for rapid, reproducible LNP screening, supporting its application in early-stage formulation development.

4.7 Supplementary Information for Publication

Flow cytometry analysis was gated based on three control groups: unstained live cells (Figure 4-11), stained live cells (Figure 4-12), and stained dead cells (Figure 4-13). Once gating was appropriate for all three controls, sample analysis was undertaken.

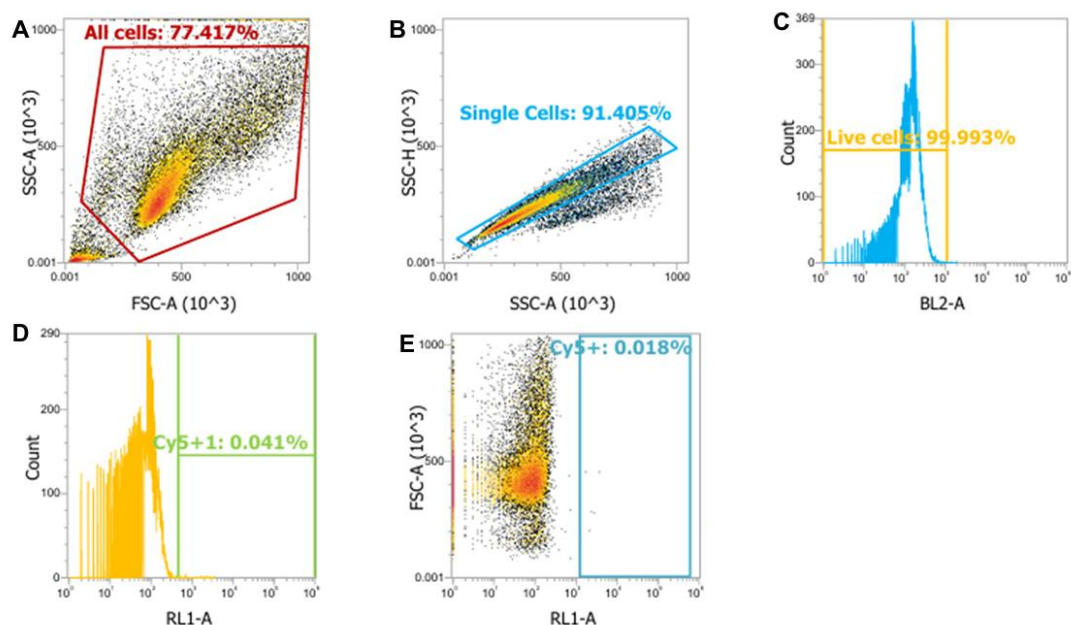


Figure 4-11 Gating Strategy for unstained live cell control. Gating strategies were set up using an unstained live cell control (shown below) before being used for positive and negative controls as well as sample runs. (A) All Cells gate (red) was set to contain the live and healthy cell population and exclude any dead cells or possible cell debris as indicated by very high or low FSC-A and SSC-A (B) single cells gate (blue) were selected to exclude cells populations that were possibly stuck together as indicated by an increase in one of either side scatter area or height (C) BL2 voltages were adjusted until complete signal seen on the left hand side of the panel within live cells gate (yellow) indicating cell viability as propidium iodide stains dead cells while (D) RL1 voltages were adjusted until Cy5+ signal gate (green) had minimal signal remaining on the right hand side of panel, finally (E) represents Cy5+ signal as a function of forward scattering.

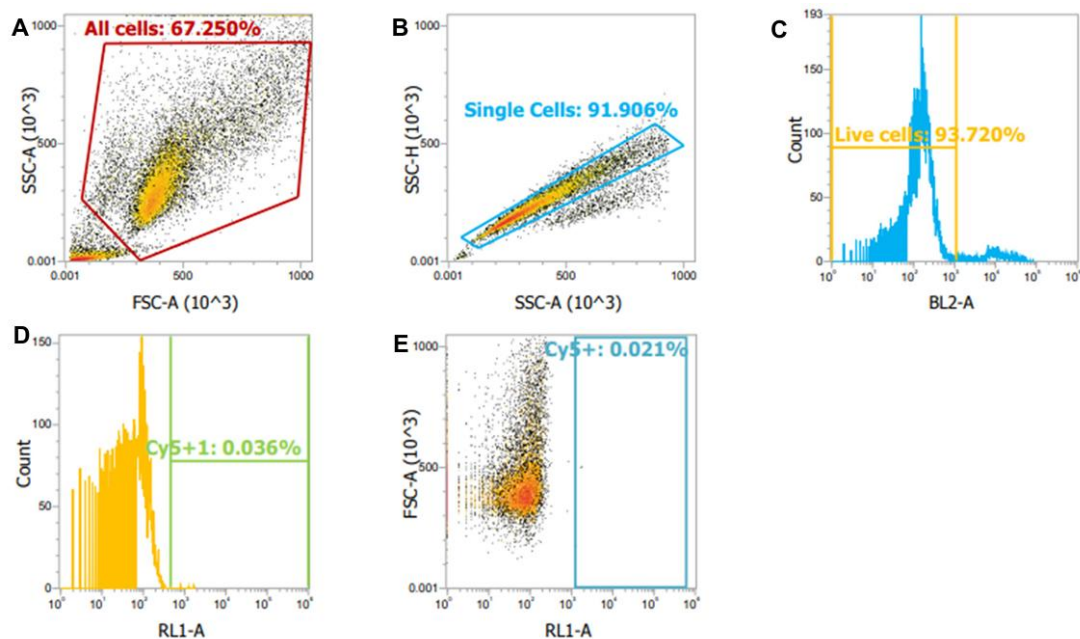


Figure 4-12 Gating strategy for stained live cell control. Gating strategies were established using a stained live cell control (shown below) prior to sample analysis. All gates (A–E) were applied as described in Figure S1, using these control samples to confirm that the cell population remained viable following sample preparation. This ensured that results were representative of a live, healthy cell population.

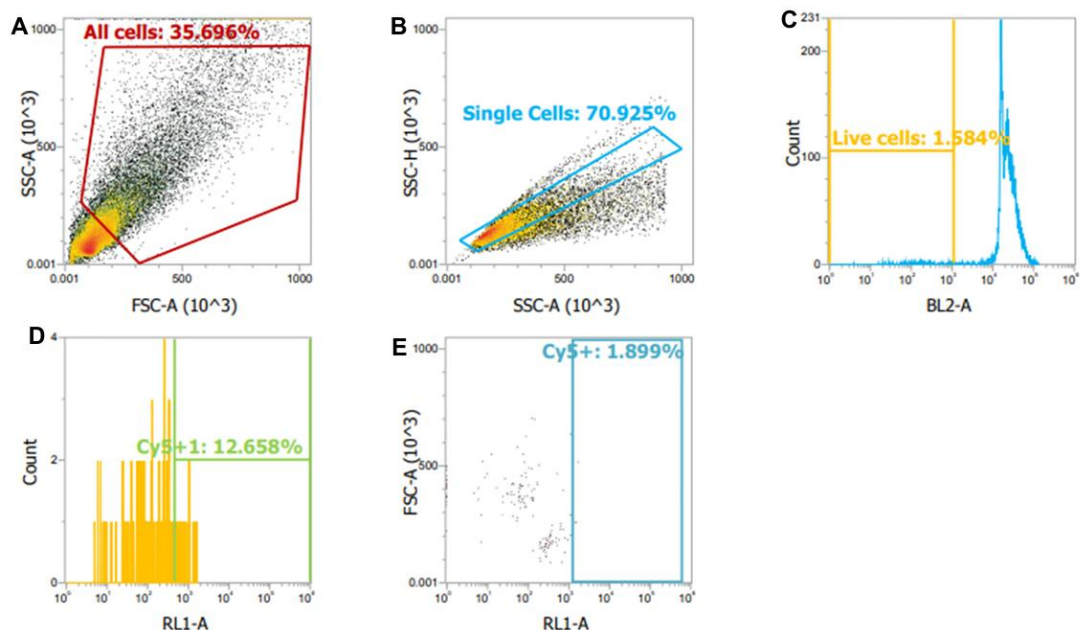
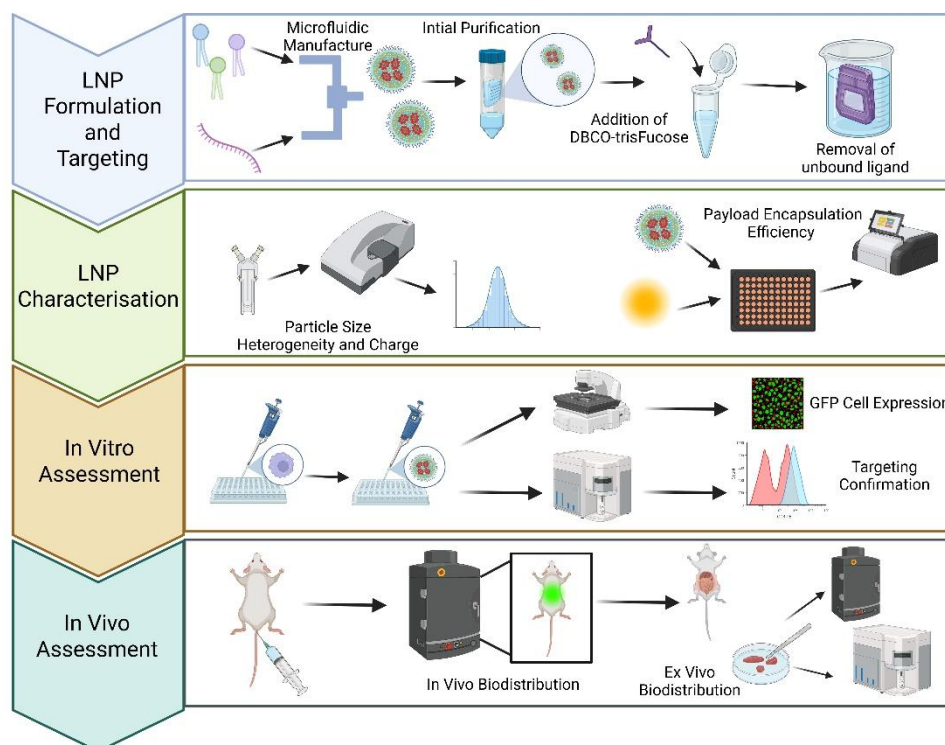


Figure 4-13 Gating Strategy for stained dead cell control. Gating strategies were set up using a stained dead cell control (shown below) before being used for sample runs. All gates (A–B, D–E) were set up as described for Figure S1 using these dead cell samples to help ensure that the gating was correct and ensure viability. For the live cells gate (yellow) (C), all signals should be present on the right-hand side of the panel due to the propidium iodide signal being positive and staining dead cells.

Chapter 5: First Responders Meet First Class Delivery: LNPs for Macrophage Targeting

Type of Publication	☐ Published article ☐ Accepted for publication ☐ Submitted for publication ☒ Drafted for publication/application notes ☐ Not applicable
Citation Details (only applicable if published)	N/A
Chapter Inclusion Details	☒ This chapter is a drafted version of a manuscript for submission ☐ This chapter contains minor revision of the original publication ☐ This majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	CRediT Conceptualisation was carried out by J.F., Y.P., and A.M. Methodology was designed by J.F., Y.P., G.V., D.W., and A.M. Investigation was conducted by J.F., Y.P., G.V., D.W., and A.M. Data curation was overseen J.F., Y.P., and A.M. Preparation of original draft was undertaken by J.F., and Y.P. Visualisation was handled by J.F., and Y.P.



5.1 Abstract

Introduction: Lipid nanoparticles (LNPs) are a clinically validated nucleic acid delivery system; however, standard formulations generally lack a cell-type specific targeting ability, resulting in non-selective delivery and increased off-target effects. In an attempt to improve targeting specificity, researchers have begun leveraging uptake routes and/or specific cell-surface receptors to improve the delivery of nucleic acids. In this study, we aim to target the M2-macrophage subtype by conjugating a DBCO-trisFucose targeting ligand to the surface of commercially available LNP formulations. The targeting ligand is specifically designed to bind the CD206 receptor, which is commonly overexpressed on the surface of M2 macrophages. Successful delivery of LNPs could enhance modulation of immune responses, potentially improving disease outcomes.

Methods: Control LNPs were manufactured using a standard lipid mix containing DSPC, cholesterol, SM-102 or DLin-MC3-DMA and DMG-PEG2000 or DSPE-PEG2000, while targeted LNPs also incorporated varying proportions of

DMG/DSPE-PEG2000-Azide to enable click reactions with the DBCO-trisFucose targeting ligand through Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC). All formulations underwent physicochemical characterisation to evaluate LNP size, homogeneity, charge and encapsulation. LNP efficacy was evaluated using a murine macrophage cell line, alongside *in vivo* biodistribution studies. **Results:** Addition of the targeting ligand had minimal effect on formulations CQAs with sizes between 74-110 nm, PDI values <0.2, and encapsulation efficiencies >83%. Initially, we demonstrated that SM-102-based formulations exhibited higher *in vitro* protein expression than DLin-MC3-DMA-based formulations. Furthermore, standard *in vitro* protein expression protocols showed that the majority of formulations increased expression in RAW264.7 cells stimulated with IL-4 to enhance M2 populations, as assessed by flow cytometry. This indicates that the LNP formulation with 100% surface-targeting modification preferentially transfects CD206+/GFP+ cells, demonstrating receptor selectivity *in vitro*. No significant effects on cell viability were observed. Finally, using *in vivo* models, it was demonstrated that the LNP formulation with 50% surface targeting showed comparable whole-mouse expression to the standard non-targeted formulations. **Conclusions:** Overall, this study has established a strong foundation for macrophage-targeted LNPs, demonstrating receptor-selective targeting leading to increased protein expression *in vitro*. Furthermore, targeted LNPs demonstrate consistent, similar *in vivo* expression to non-targeted LNPs, indicating that formulation modifications do not compromise *in vivo* efficacy.

5.2 Introduction

Macrophages are a functionally diverse cell type within the innate immune system and have a large effect on disease progression, maintenance of homeostasis and growth and repair [209, 210]. Macrophages are generally present in tissues and are derived from mature monocytes in the blood [210]. After this, they will exhibit increased levels of heteroplasticity maturing into either

M1 or M2, depending upon stimuli in the tissue microenvironment [209]. In their resting state, macrophages exist as M0, and upon stimulation with LPS or IFN- γ they polarise into M1, whereas stimulation with IL-4 or IL-13 causes M2 polarisation [211, 212]. Notably, polarisation with IL-4/IL-13 results in M2a macrophages specifically, whereas M2b, M2c, and M2d subsets can be induced by alternative stimuli [203], again indicating the high levels of heteroplasticity that macrophages exhibit. A diagram detailing M0 polarisation is shown in Figure 5-1.

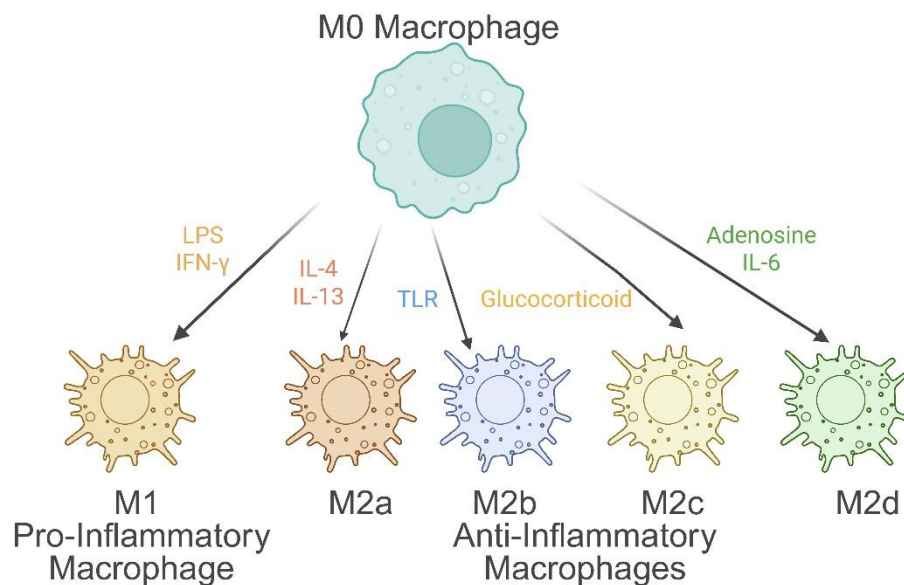


Figure 5-1 Polarisation of M0 Macrophages. This diagram shows the stimuli required to polarise M0 macrophages into M1 and M2 subtypes. LPS = Lipopolysaccharide (LPS), IFN- γ = Interferon Gamma, IL = Interleukin, TLR = Toll-Like Receptors. The figure was prepared using BioRender.

M1 macrophages are the classically activated phenotype and are generally known as pro-inflammatory macrophages due to their role in producing Reactive Oxygen Species, which are toxic to pathogens [209, 212]. On the other hand, M2 macrophages are detailed as the alternatively activated pathway and are key phagocytic drivers, as well as playing key roles in promoting wound healing and tissue repair [209, 211]. When the body is challenged with an unknown pathogen or injury, the innate immune response will initiate inflammation pathways. These

pathways are tightly regulated, and prolonged or insufficient responses result in M1 and M2 imbalances, which can increase the risk of autoimmune disease [211].

Within the tumour microenvironment, cells which exhibit M2 phenotypes are often termed as Tumour Associated Macrophages (TAMs) [213] as they are key contributors to tumour progression and hence resistance to any potential treatments [213]. TAMs are thought to be reprogrammed by tumour-derived growth factors [214]. When this shift occurs, M2 macrophages will begin to support tumour progression. TAMs have been shown to promote neovascularisation and cause the secretion of pro-angiogenic factors like platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which can facilitate tumour cell metastasis [212]. However, it is important to note that TAMs can play positive roles in cancer following re-education into a more M1-phenotype, offering anti-inflammatory properties [215]. These key abilities make macrophages, specifically TAMs, promising targets.

Due to an M2 phenotype being most abundant for TAMs, many of the macrophages will possess the mannose receptor, commonly known as the CD206 receptor [211]. The CD206 receptor has been a target of many research articles, with down regulation of the CD206 receptor in bone marrow-derived macrophages shown to inhibit breast cancer growth in a murine model [213]. Furthermore, high levels of CD206 have been shown to account for poor prognosis in laryngeal cancer [216], solid malignancies [217] and intrahepatic cholangiocarcinoma [218] to name a few. This highlights the feasibility of the CD206 receptor as a target for initial tumour growth inhibition or offering immune response modulation. The CD206 receptor is part of the C-type lectin family, which generally binds glycans and sugars on pathogen targets [219, 220]. The receptor itself contains eight C-type lectin-like domains, allowing it to bind fucose, mannose or *N*-acetylglucosamine (GlyNac) [221]. The current

macrophage targeted LNP landscape is focused on active targeting of the formulation. For example, Kim et al., demonstrated increased uptake and mRNA delivery in CD206+ liver cells using an LNP containing a mannose-conjugated cholesterol [222]. Similarly, Zhao et al., manufactured a surface modified LNP (MacLNP) containing the macrophage marker F4/80 and they were able to demonstrate significant enhancing of macrophage targeting [223]. Subsequently, other delivery vehicles are also being investigated for macrophage targeting, with a high number utilising mannose as their targeting receptor. For example, Li et al. used mannose-conjugated Iron Oxide Nanoparticles to perform targeted imaging of the mannose receptor [224]. Interestingly, Morimoto et al. investigated immune checkpoint receptor depletion in M2 macrophages using tumour immune cell targeting chimaeras (TICTACs), which are conjugated to several receptors targeting CD206 [225]. These receptors include fucose-, galactose-, and mannose-targeted ligands, with all receptors demonstrating target downregulation [225]. Building on this research, we aimed to establish a novel M2 macrophage therapeutic by employing commercially available LNPs functionalised with a CD206-targeting ligand. We opted to conjugate our targeting ligand onto the surface of an LNP due to the fact that they often lack a cell-specific delivery, and alteration of the lipid composition is often required to target these formulations [156]. Hence, we synthesised a DBCO-trisFucose ligand, which we aimed to conjugate to LNP formulations via PEG-Azide. When present together, DBCO and Azide will conjugate via a click chemistry reaction known as strain-promoted azide-alkyne cycloaddition (SPAAC), resulting in a fast, specific reaction without the need for a catalyst. Additionally, the reaction can be carried out under aqueous conditions, making it particularly advantageous for LNPs [226].

5.3 Materials and Methods

5.3.1 Materials

The ionizable lipids Heptadecan-9-yl 8-((2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino) octanoate (SM-102) and (6Z, 9Z, 28Z, 31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA) were purchased from Broadpharm (San Diego, CA, USA) or supplied by R&D Systems (Bio-Techne, Bristol, UK). 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG₂₀₀₀), 1,2-dimyristoyl-rac-glycero-3-(azidopolyethylene glycol-2000) (DMG-PEG₂₀₀₀-Azide), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were sourced from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) alongside, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carbonyl azido(polyethyleneglycol)-2000] (DSPE-PEG₂₀₀₀-Azide), DBCO-trisFucose, RiboDyEE™ and murine recombinant IL-4 protein were provided by R&D Systems (Bio-Techne, Bristol, UK). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol 2000 (DSPE-PEG₂₀₀₀) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol and heat-inactivated fetal bovine serum (FBS) were bought from Merck Life Science UK Ltd (Gillingham, UK), while Ethanol (HPLC-grade) was purchased from Supelco (Bellefonte, PA, USA). Sodium citrate buffer, Tris-HCL buffer, Diethylpyrocarbonate (DEPC), Triton X-100, 20X TE-buffer, Invitrogen™ CD206 (MMR) Monoclonal Antibody (MR6F3) APC, Molecular Probes™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit and CD16/CD32 Rat anti-mouse were purchased from Thermo Fisher Scientific (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Cell culture materials, including Phenol Red Free Dulbecco's Minimal Essential Media (DMEM), DMEM Glutamax, sodium pyruvate, penicillin, streptomycin, DBPS and TrypLE, were acquired from Gibco Technologies (Thermo Fisher Scientific, Waltham, MA, USA). The VivToGlo™ Luciferin was purchased from Promega Ltd (Chilworth, UK),

and the Fluc-Fusion and Green Lantern mRNAs were supplied by CPI (Centre for Process Innovation, Sedgefield, UK). Ultrapure water was supplied through an in-house system. RAW264.7 cells were purchased from American Type Culture Collection (ATCC) (TIB-71).

5.3.2 Chemistry of Ligand Synthesis

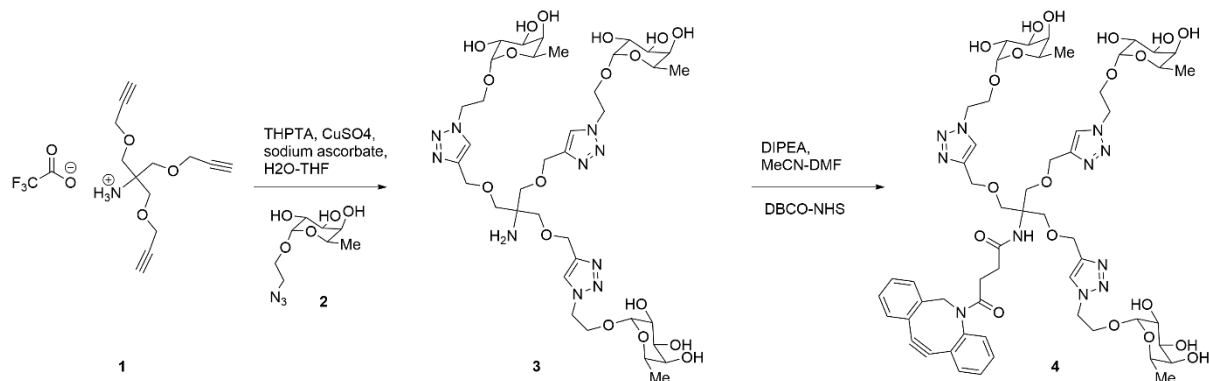


Figure 5-2 Chemical Synthesis of DBCO-trisFucose.

All reagents and solvents were purchased from commercial sources and used without further purification. Nuclear magnetic resonance spectra were recorded on a Bruker Avance III HD spectrometer operating at 400 MHz for ¹H NMR. ¹H NMR chemical shifts (δ) are reported in parts per million (ppm) and are referenced to residual protium in solvent. Purification by prep-RP-HPLC was performed using an Agilent 1260 Infinity II system with a Waters Atlantis T3, 5 μm, 19 x 150 mm column. Reaction monitoring LCMS analyses were conducted using Agilent InfinityLab LC/MSD systems.

Abbreviations: DBCO (dibenzocyclooctyne), THPTA (tris-hydroxypropyltriazolylmethylamine), NHS (*N*-hydroxysuccinimide), DIPEA (*N,N*-diisopropylethylamine).

1,3-Bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-amine, trifluoroacetate salt (1);

tert-Butyl (1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)carbamate (890 mg, 2.52 mmol) was dissolved in DCM (9 mL), and trifluoroacetic acid (4.6 mL, 6.98 g, 61.3 mmol) was added in a dropwise fashion

over 2 minutes. After stirring at room temperature for 2 hours, the solvent was removed *in vacuo* and the residue coevaporated with dichloromethane (3 x 20 mL) to give 1,3-*bis*(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-amine, trifluoroacetate salt (**1**), a brown oil (0.40 g, 1.15 mmol, 45%).

¹H NMR (400 MHz, CDCl₃), δ 7.91 (br s, 2H), 4.22 (d, 6 H, $J=2.2$ Hz), 3.75 (s, 6 H) and 2.50 (t, 3 H, $J=2.2$ Hz).

Tris-fucose amine, formate salt (3);

A solution of THPTA (130 mg, 0.30 mmol), copper sulfate.5H₂O (37 mg, 0.15 mmol) and sodium ascorbate (89 mg, 0.45 mmol) in water (1 mL) was added to a solution of 1,3-*bis*(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-amine, trifluoroacetate salt (**1**) (50 mg, 0.14 mmol) and 2-azidoethyl α -L-fucopyranoside (**2**) (139 mg, 0.60 mmol) in THF/water (1:1, 2.5 mL). After stirring at room temperature overnight, the solvent was removed *in vacuo*, and the residue was purified using prep-RP-HPLC (5-20% acetonitrile/0.1% formic acid (aq) gradient over 15 min). Product fractions were combined and lyophilised to give *tris*-fucose amine, formate salt (**3**), a white solid (50 mg, 0.051 mmol, 36%). This material was taken forward for the synthesis (**4**).

LC-MS (m/z): Calcd for [M-H]⁻: 933.4, Found: 933.2.

Tris-fucose-DBCO (4);

Tris-fucose amine.formate (**3**) (50 mg, 0.051 mmol) was dissolved in acetonitrile-DMF (2-1, 3 mL). DIPEA (44 μ L, 0.25 mmol) was added, followed by DBCO-NHS (25 mg, 0.061 mmol), and the reaction was stirred at room temperature overnight. LCMS showed an incomplete reaction. DBCO-NHS (37 mg, 0.091 mmol) was added, and stirring was continued at room temperature overnight. The solvent was removed *in vacuo*, and the residue was purified using prep-RP-HPLC (10-100% acetonitrile/0.1% formic acid (aq) gradient over 15 min). Product fractions

were combined and lyophilised to give *tris*-fucose-DBCO (**4**) as a white solid (17 mg, 0.014 mmol, 27%).

¹H NMR (400 MHz, CD₃OD), δ 8.06 (s, 3H), 7.64-7.58 (m, 2H), 7.49-7.42 (m, 3H), 7.34-7.27 (m, 2H), 7.26-7.20 (m, 1H), 5.13 (d, *J*=14.0 Hz, 1H), 4.75 (d, *J*=3.8 Hz, 3H), 4.69-4.55 (m, 6H), 4.50 (s, 6H), 4.04 (ddd, *J*=11.2, 8.2 & 3.8 Hz, 3H), 3.82 (dt, *J*=10.1 & 4.1 Hz, 3H), 3.72 (dd, *J*=10.2 & 3.7 Hz, 4H), 3.68-3.59 (m, 9H), 3.55 (d, *J*=2.8 Hz, 3H), 3.36 (q, *J*=6.8 Hz, 3H), 2.65 (ddd, *J*=16.4, 7.6 & 2.0 Hz, 1H), 2.39 (dt, *J*=15.3 & 7.4 Hz, 1H), 2.20 (dt, *J*=14.6 & 6.7 Hz, 1H), 1.94 (dt, *J*=16.0 & 6.7 Hz, 1H) and 1.08 (dd, *J*=6.4 & 1.2 Hz, 9H).

LC-MS (*m/z*): Calcd for [M+Na]⁺: 1244.5, Found: 1244.4. Calcd for [M-H]⁻: 1220.5, Found: 1244.3.

Method modified from [225].

5.3.3 Lipid Nanoparticle Formulation

For standard LNPs, the lipid phase consisted of DSPC, cholesterol, the ionizable lipid (SM-102 or DLin-MC3-DMA) and the PEGylated lipid (DMG-PEG2000 or DSPE-PEG2000) at a molar ratio of 10.9:42:45.5:1.6% dissolved in absolute ethanol (>99.5%). The lipid phase of targeted LNPs also incorporated DMG-PEG2000-Azide or DSPE-PEG2000-Azide at molar ratios of 0.3%, 0.8% or 1.6%, making up a molar ratio of 10.9:42:45.5:1.3:0.3%, 10.9:42:45.5:0.8:0.8% or 10.9:42:45.5:1.6%, respectively. The aqueous phase for all LNPs contained Fluc mRNA or Green Lantern mRNA, diluted in 50 mM citrate buffer at pH 5 to achieve a nitrogen:phosphate (N/P) ratio of 6. LNPs were manufactured on the Nova Benchtop™ (Helix Biotech, TN, USA) using either the size 1 mixer at a total flow rate (TFR) of 15 mL/min or the size 0 mixer at a TFR of 10 mL/min. Flow rate ratios were held constant at 3:1 (aqueous:lipid).

5.3.4 Targeted Ligand Conjugation

Initial conjugation trial protocols resulted in DBCO-trisFucose being mixed with the LNPs samples post-manufacture at a ratio of 3:1 (DBCO-trisFucose:initial PEG-Azide concentration) with condition 1 and condition 2 being conjugated at 4 degrees for 24 hours, and condition 3 and condition 4 being conjugated at room temperature for 24 hours. Conditions 2 and 4 were subsequently purified by dialysing the sample against 200 mL of 10 mM Tris buffer (pH 7.5) for 1 hour at 4 degrees. For the remainder of the study, condition 2 was chosen for subsequent conjugations, but dialysis time was increased to 4 hours.

5.3.5 Lipid Nanoparticle Purification

Post-manufacture purification was carried out using spin column chromatography. LNP samples were diluted 1:10 with 10 mM Tris buffer (pH 7.5), and centrifugal filtration was carried out using Amicon Ultra-15 columns at 2000 × g for 4 degrees until the original volume was reached. At this point, the LNPs were further diluted 1:5 with 10 mM Tris buffer (pH 7.5), and filtration was performed again until the original volume was reached. To remove any unbound DBCO-trisFucose, LNP samples were dialysed against 200 mL of 10 mM Tris buffer (pH 7.5) for 4 hours at 4°C using dialysis tubing with a molecular weight cut-off of 14 kDa.

5.3.6 Lipid Nanoparticle Critical Quality Attribute Testing

Lipid nanoparticle z-average diameter (nm), polydispersity index and zeta potential (mV) were monitored at a lipid concentration of 0.1 mg/mL using the Zetasizer Ultra equipped with a 633 nm laser and a back scatter detection angle of 173° (Malvern Panalytical, Ltd., Worcestershire, UK). Pre-purification measurements were carried out by diluting the samples in 50 mM citrate buffer at pH 5 (viscosity 1.28 cP and refractive index 1.47). Post-purification measurements were carried out in 10 mM Tris buffer at pH 7.5 (viscosity 1.0037 cP and refractive index 1.337). For Zeta potential measurements, LNPs were

diluted in water (viscosity 0.8872 cP and refractive index 1.33). Z-average diameter (nm) and polydispersity index were measured in a ZEN0040 cuvette, and zeta potential (mV) was measured in a DTS1070 cuvette.

5.3.7 Payload Encapsulation Quantification

The encapsulation efficiency (%) and mass balance (%) (mRNA recovery) were assessed using a Ribogreen™ or RiboDyEE™ Assay. LNPs were diluted to concentrations of 3 µg/mL in 1X Tris-EDTA buffer, and 50 µL of LNP was added to wells in duplicate or triplicate wells with either 50 µL of 1X Tris-EDTA buffer or 50 µL of 2% Triton-1X Tris-EDTA buffer. Two standard curves were prepared for analysis: a high curve (0-1000 ng) for Triton (+) wells or a low curve (0-50 ng) for Triton (-) wells. The assay plate was then incubated for 15 minutes at 37°C to allow ‘bursting’ of LNPs, and total and free mRNA were quantified through the addition of 100 µL of either 500X (Triton (-) wells) or 200X (Triton (+) wells) of Ribogreen™ or RiboDyEE™. The fluorescence was quantified using the GloMax® microplate reader (Promega, Chilworth, UK) at excitation 475 nm and emission 500-550 nm.

$$\text{Encapsulation efficiency (\%)} = \left(\frac{\text{Total RNA} - \text{Free RNA}}{\text{Total RNA}} \right) \times 100\%$$

$$\text{Mass balance (\%)} = \left(\frac{\text{Total RNA}}{\text{Theoretical RNA}} \right) \times 100\%$$

5.3.8 Cell Viability

RAW264.7 cells were seeded in a clear 96-well plate at a seeding density of 1.5×10^4 cells/well in Phenol Red (PR) Free DMEM Glutamax supplemented with 10% FBS and 1% Penicillin Streptomycin. For polarised cells, murine IL-4 recombinant protein was added at seeding at a concentration of 25 ng/mL. Cells were cultured at 37°C, 5% CO₂ for 48 hours. LNP treatment was performed at mRNA concentrations of 2, 1, 0.5, and 0.25 µg/mL for 24 or 48 hours at 37°C and 5% CO₂. Following incubation, 10 µL of resazurin was added to each, and the cells

were incubated for a further 4 hours at 37°C, 5% CO₂. Fluorescence was then read on the GloMax® microplate reader (Promega, Chilworth, UK) at excitation 520 nm and emission 580-640 nm.

$$Cell\ Viability\ (\%) = \left(\frac{Treated}{Untreated\ Control} \right) \times 100\%$$

5.3.9 Green Lantern mRNA Expression *In vitro*

RAW264.7 cells were seeded in a black clear-bottom 96-well plate at a seeding density of 1.5×10^4 cells/well in Phenol Red (PR) Free DMEM Glutamax supplemented with 10% FBS and 1% Penicillin Streptomycin. For polarised cells, murine IL-4 recombinant protein was added at seeding at a concentration of 25 ng/mL. Cells were cultured at 37°C, 5% CO₂ for 48 hours. LNP treatment was carried out at mRNA concentrations of 2, 1, 0.5 and 0.25 µg/mL for 24 or 48 hours at 37°C, 5% CO₂. Following treatment, green fluorescent protein (GFP) expression was visualised on the EVOS M5000 microscope (Thermo Fisher Scientific, Waltham, MA, USA) using the Trans and GFP filters with a 10X objective. For imaging, the gain was fixed at 4, and the LED and exposure were adjusted as needed for each repeat. GFP expressed was quantified from images using ImageJ software v1.54.

$$\begin{aligned} &Cell\ Total\ Corrected\ Fluorescence\ (CTCF) \\ &= Integrated\ Density\ of\ GFP\ Image \\ &- (Area\ of\ GFP\ Image \times Mean\ of\ Blank\ GFP\ Wells) \end{aligned}$$

5.3.10 Confirmation of Targeting Selectivity Using Flow Cytometry

RAW264.7 cells were seeded in a clear 12-well plate at a seeding density of 1.5×10^5 cells/well in Phenol Red (PR) Free DMEM Glutamax supplemented with 10% FBS and 1% Penicillin Streptomycin. For polarised cells, murine IL-4 recombinant

protein was added at seeding at a concentration of 25 ng/mL. Cells were cultured at 37°C, 5% CO₂ for 48 hours. LNP treatment was carried out at an mRNA concentration of 2µg/mL for 24 hours at 37°C, 5% CO₂.

On the day of analysis, spent media was removed from wells, 1mL of DPBS was added, and plates were incubated on ice for 4 minutes. Wells were then scraped to collect cell suspension, and subsequent suspensions were centrifuged for 5 minutes at 300 x g until a cell pellet was observed. The supernatant was removed, and cell pellets were resuspended in DPBS. Dead-cell controls were obtained by incubating cells at 95 °C for 5 minutes. 0.5 µL of Fixable Violet Live/Dead™ stain was added to the appropriate samples, which were then incubated at room temperature for 30 minutes. Following staining, 1 mL of DPBS was added, and cells were centrifuged again for 5 minutes at 300 × g to form a pellet. Pellets were resuspended in 100 µL antibody mixer containing 0.25 µL/test of Fc Block (CD16/CD32 Rat Anti-Mouse) and 1.25 µL/test of CD206-APC in antibody dilution buffer and stained for 25 minutes. A final wash step was performed by adding 1 mL of DPBS, and cells were again centrifuged for 5 minutes at 300 × g to obtain a pellet, which was resuspended in DPBS and analysed immediately. Flow cytometry analysis was conducted using the Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) with LIVE/DEAD monitored using the VL1 laser, while CD206-APC was monitored on RL1, and GFP was on BL1. Gating was confirmed using a live unstained control, a live stained control and a dead control, which were monitored with each individual run.

5.3.11 *In vivo* Biodistribution Studies

16-weeks old female BALB/c mice were injected intravenously (I.V.) with 50 µL of Fluc mRNA-LNP solution equating to 1 µg mRNA/mouse. Imaging was conducted at 6 h, 24 h, and 48 h, and on days 5, 7, 14, and 21 post-injection, by subcutaneously injecting mice with D-luciferin (30 mg/mL in sterile DPBS) at a dose of 5 µL/g. 10 minutes post-injection, images were acquired in both dorsal

and ventral positions to measure bioluminescence signals using the IVIS® Spectrum (Revvity Inc., Waltham, MA, USA). Mice were also subjected to micro-CT imaging at the 6-hour time point, using only the Quantum GX2 micro-CT imaging system (Revvity Inc., Waltham, MA, USA). Imaging was conducted immediately following DLIT imaging on the IVIS® Spectrum, and the acquisition settings were as follows: voltage = 90 kV; current = 88 μ A; radiation dose = 24 mGy; Fov = 72 mm; Voxel size = 144 μ m; X-ray filter = Cu 0.06+Al 0.5; scan time = 8 seconds x 3. Images were compiled and analysed using Living Image 4.0 software.

5.3.12 *In vivo* Targeted Specificity

16-weeks old BALB/c mice were injected intravenously with 1 μ g mRNA/mouse or 7 μ g mRNA/mouse of GrL-LNPs into the tail vein. 24 hours post-injection, mice were humanely culled, and the heart, liver, spleen, lungs, and one kidney were harvested for ex vivo imaging using the IVIS® Spectrum (Revvity Inc., Waltham, MA, USA). Following imaging, the liver and spleen were diced, and the spleen was placed in 2 mL of DBPS, while the liver was placed in 2 mL of DBPS containing 200 U of Collagenase II for 2 hours at 37 °C. Following incubation, both the spleen and liver were passed through cell strainers, and the collected solution was centrifuged at 300 $\times$ g for 5 minutes at 4 °C. Afterwards, the supernatant was removed, and the pellet was resuspended in 1 mL of DPBS and spun again. The pellet was then resuspended in 1 mL of DPBS, and a further 1:10 dilution was undertaken. 0.5 μ L of Fixable Violet LIVE/DEAD Stain was added to the diluted cell suspensions, and the mixtures were incubated at room temperature for 30 minutes. Cells were washed and spun as per the previous wash, and the pellet was resuspended in 100 μ L of antibody mixer containing 0.25 μ L/test of Fc Block (CD16/CD32 Rat Anti-Mouse) and 1.25 μ L/test of CD206-APC in antibody dilution buffer, then stained for 25 minutes. A final wash step was performed by adding 1 mL of DPBS, and cells were again centrifuged for 5 minutes at 300 $\times$ g to obtain a pellet, which was resuspended in DPBS and analysed immediately. Flow

cytometry analysis was conducted using the Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) with LIVE/DEAD monitored using the VL1 laser, while CD206-APC was monitored on RL1, and GFP was on BL1.

5.3.13 Ethics Statement

Animal experiments and experimental procedures were carried out in accordance with UK Home Office regulations and the University of Strathclyde Animal Welfare and Ethical Review Board Regulations under project license number PPL PP1650440. BALB/c mice were bred and maintained in the Biological Procedures Unit at the University of Strathclyde, Glasgow, and experiment design and reporting adhered to the ARRIVE guidelines.

5.3.14 Statistical Analysis

All results represent the mean $\pm$ standard deviation (physicochemical analysis) or standard error of the mean (*in vitro*/*n vivo*). All results were checked for normality using an Anderson-Darling normality test. Subsequent parametric data were analysed using an ANOVA following Tukey's post hoc test, while non-parametric data were analysed using a Kruskal-Wallis test followed by Dunn's post hoc test.

5.4 Results

5.4.1 Determination of Optimal Ligand Conjugation Conditions

The click chemistry reaction between DBCO and azide groups is well documented in literature and is reported to occur rapidly and efficiently at room temperature. However, LNP stability is improved at refrigerated temperatures (2-8°C). Therefore, we aimed to determine the optimal binding condition that balances LNP stability and ligand-binding potential.

Four binding conditions were investigated; conditions 1 and 2 were conducted at refrigerated temperatures, while conditions 3 and 4 were conducted at room temperature. Conditions 2 and 4 were subsequently dialysed to remove any remaining DBCO-trisFucose. *In vitro* protein expression and cell viability were also monitored in a stably transfected cell line (HEK-293). The results, along with LNP physicochemical analysis, are shown in Figure 5-3.

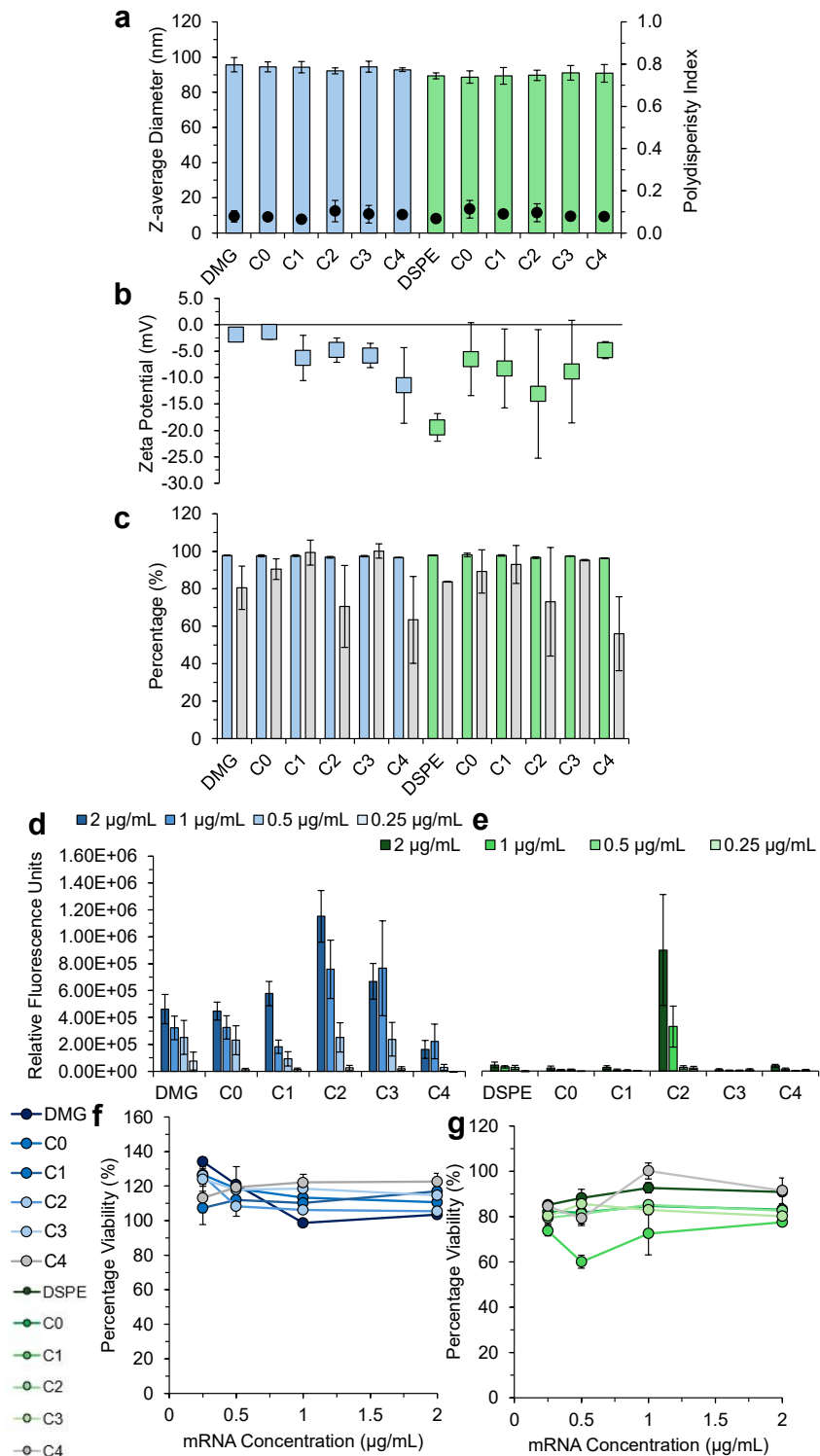


Figure 5-3 Optimisation of DBCO-trisFucose Binding Conditions. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:1.3:0.3%) encapsulating Green Lantern mRNA at a final concentration of 0.04 mg/mL. LNPs were either representative of a control formulation (DMG/DSPE), Azide-containing LNP with no DBCO-trisFucose (C0), DBCO-trisFucose conjugated at refrigerated temperatures (C1/C2) or room temperature (C3/C4), with C2/C4 also undergoing dialysis to remove additional DBCO-trisFucose. (a) represents z-average diameter (nm) (bars) and polydispersity index (circles) while (b) represents the zeta potential (mV) and (c) represents the encapsulation efficiency (%) (coloured bars) and mass balance (grey bars). LNPs were used to treat HEK-293 cells, and 48 post-transfection Green Fluorescent Protein (GFP) expression (d/e) and cell viability for both DMG-based LNPs (f) and DSPE-based LNPs (g) were monitored. Results are representative of the mean $\pm$ standard deviation (a-c) or standard error of the mean (d-g) (n=2).

All tested ligand-binding conditions resulted in no significant changes in the physicochemical characterisation of the LNP formulations (Figure 5-3a-c), with LNP sizes ranging from 88-95 nm, PDI < 0.12, and zeta potentials ranging from -1 to -12 mV. Encapsulation efficiency of mRNA ranged between 96-98% while mass balance ranged between 56-100% (Figure 5-3c). The lower mass balance observed in C2 and C4 for all LNP formulations could be attributed to decreased concentration due to swelling of the dialysis bag.

Following LNP treatment, GFP expression was monitored in HEK-293 cells (Figure 5-3d/e). Overall, no significant differences were seen between different binding conditions for either DMG- or DSPE-based LNPs ($p>0.05$). However, DMG-based LNPs exhibited higher GFP expression compared to DSPE-based LNPs (Figure 5-3d/e). Furthermore, LNPs that underwent ligand conjugation at 4 °C and then purified resulted in the highest *in vitro* GFP expression (Figure 5-3d/e). Figure 3g highlights that for DSPE-PEG LNPs, binding condition 1 resulted in a slight dip in cell viability at lower treatment concentrations, but this was not significantly different to the other DSPE-LNPs ($p>0.05$). DMG-LNPs also exhibited good cell viability with no significant differences across binding conditions (Figure 3f) ($p>0.05$).

5.4.2 Determination of Optimal *In vitro* Testing Techniques

Based on the results presented in section 5.4.1, binding condition 2 was deemed to be optimal due to increased protein expression and no significant effects on LNP CQAs. However, protein expression was initially monitored in HEK-293 cells, but these do not express the targeted CD206 receptor. Therefore, the following studies were conducted using both HEK-293 cells and RAW264.7 cells – a murine macrophage cell line – that, when stimulated with IL-4, can polarise to an M2 macrophage subtype and subsequently offer increased expression of the targeted receptor. Figure 5-4 highlights physicochemical analysis of the LNPs as well as *in vitro* GFP expression in either HEK-293 cells or RAW264.7 cells.

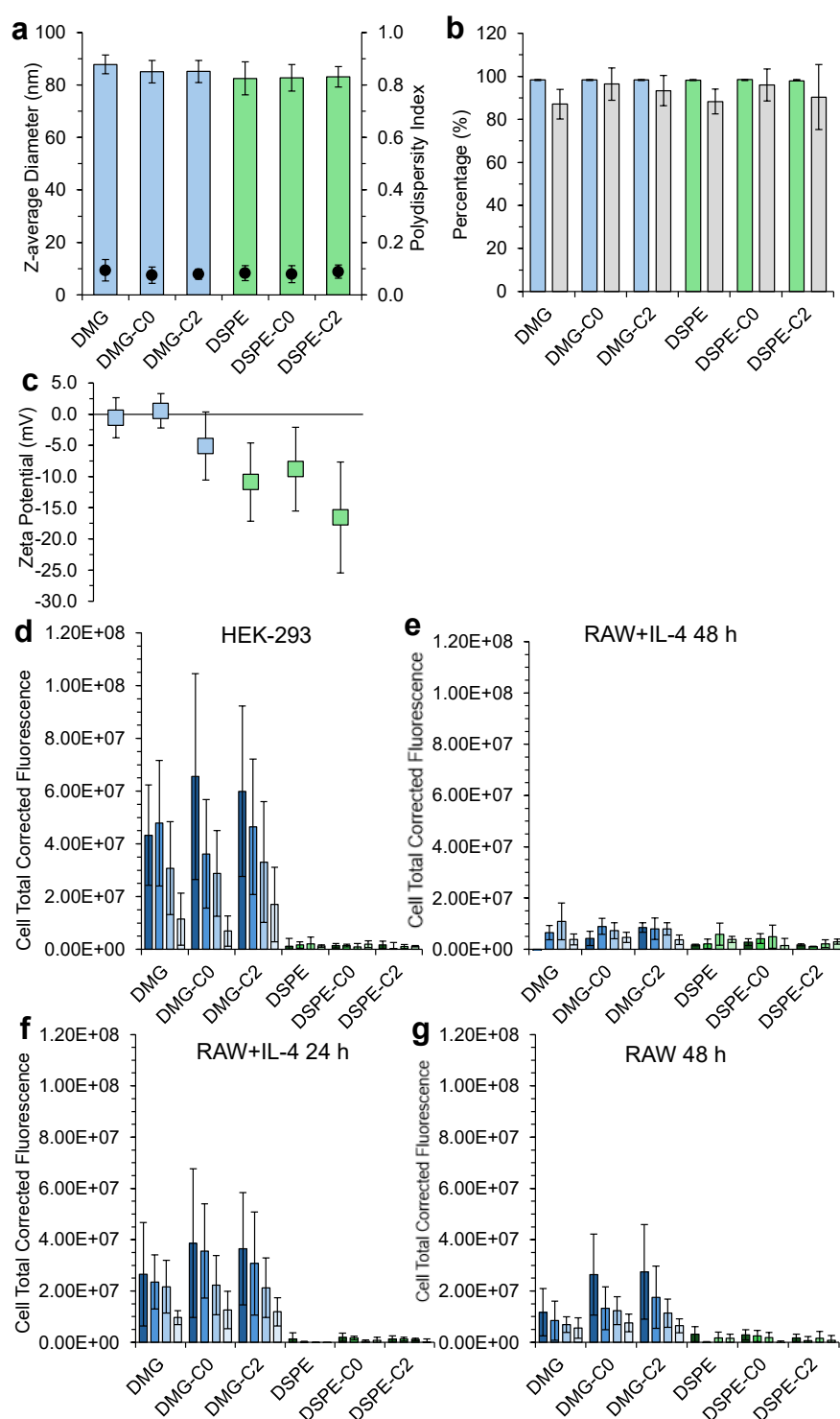


Figure 5-4 Optimisation of In Vitro Testing Conditions. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:1.3:0.3%) encapsulating Green Lantern mRNA at a final concentration of 0.04 mg/mL. LNPs were either representative of a control formulation (DMG/DSPE), Azide-containing LNP with no DBCO-trisFucose (C0), or an Azide-containing LNP with DBCO-trisFucose bound (C2). (a) represents z-average diameter (nm) (bars) and polydispersity index (circles) while (b) represents the zeta potential (mV) and (c) represents the encapsulation efficiency (%) (coloured bars) and mass balance (grey bars). LNPs were used to treat HEK-293 cells (d), RAW264.7 cells + IL-4 with 48 (e) or 24 (f) post-transfection and RAW264.7 cells after 24 hour transfection (g) Green Fluorescent Protein (GFP) expression (d-g) was monitored. Results are representative of the mean $\pm$ standard deviation (a-c) or standard error of the mean (d-g) (n=3).

Figure 5-4a/c highlights that the LNP formulations are highly reproducible post-manufacture with sizes ranging between 82-87 nm, PDI <0.1 and encapsulation efficiencies and mass balances >87%. All LNPs exhibited a slight negative shift in zeta potential upon binding of the DBCO-trisFucose from 0.5 to -5.1 mV and -8.8 to -16.5 mV for DMG- and DSPE-LNPs, respectively (Figure 5-4b). Whilst this was not significant, a clear pattern is evident. LNP formulations were then used to optimise *in vitro* testing conditions by monitoring GFP expression in HEK-293 cells (Figure 4d), RAW264.7 cells polarised with IL-4 and treated for 24 hours (Figure 5-4f) or 48 hours (Figure 5-4e) and RAW264.7 cells with no polarisation treated for 48 hours (Figure 5-4g).

Overall, Figure 5-4d-g shows that the expression patterns across the tested LNP formulations are similar regardless of testing conditions, differing only in the magnitude of GFP expression. Additionally, no significant differences in protein expression were observed among the various LNPs tested, except for DMG-LNP, which showed significantly higher expression in HEK-293 cells compared to RAW264.7 cells polarised with IL-4 and treated for 48 hours ($p < 0.01$). Figure 4e-f demonstrates that when RAW264.7 cells are polarised, expression levels are increased at a 24-hour measurement compared to that of a 48-hour measurement, as well as being increased in polarised populations compared to unpolarised populations (Figure 5-4f vs 5-4g).

5.4.3 Investigating How Altered Surface Modification Percentages Affect the LNP CQAs and Efficacy

Based on the results presented in section 5.4.2, RAW264.7 cells were selected for the remaining *in vitro* testing due to their ability to express the targeted receptor, with a treatment time of 24 hours selected due to the increased stepwise expression profiles.

While slight increases in expression were observed in polarised macrophage populations, the study next aimed to establish whether increased surface

modification affected physicochemical characterisation and to determine if higher surface modification resulted in increased protein expression. We used two commercially available and well-studied ionizable lipids (SM-102 and DLin-MC3-DMA) to manufacture DMG- and DSPE-LNPs, which were surface-modified at 0%, 0.3%, 0.8%, or 1.5%. Subsequent LNPs were used to analyse particle size, homogeneity, and encapsulation efficiency, alongside monitoring GFP expression in polarised and non-polarised macrophages *in vitro*. The results of the CQA analysis are shown in Figure 5-5, while Figure 5-6 highlights the recorded *in vitro* GFP expression.

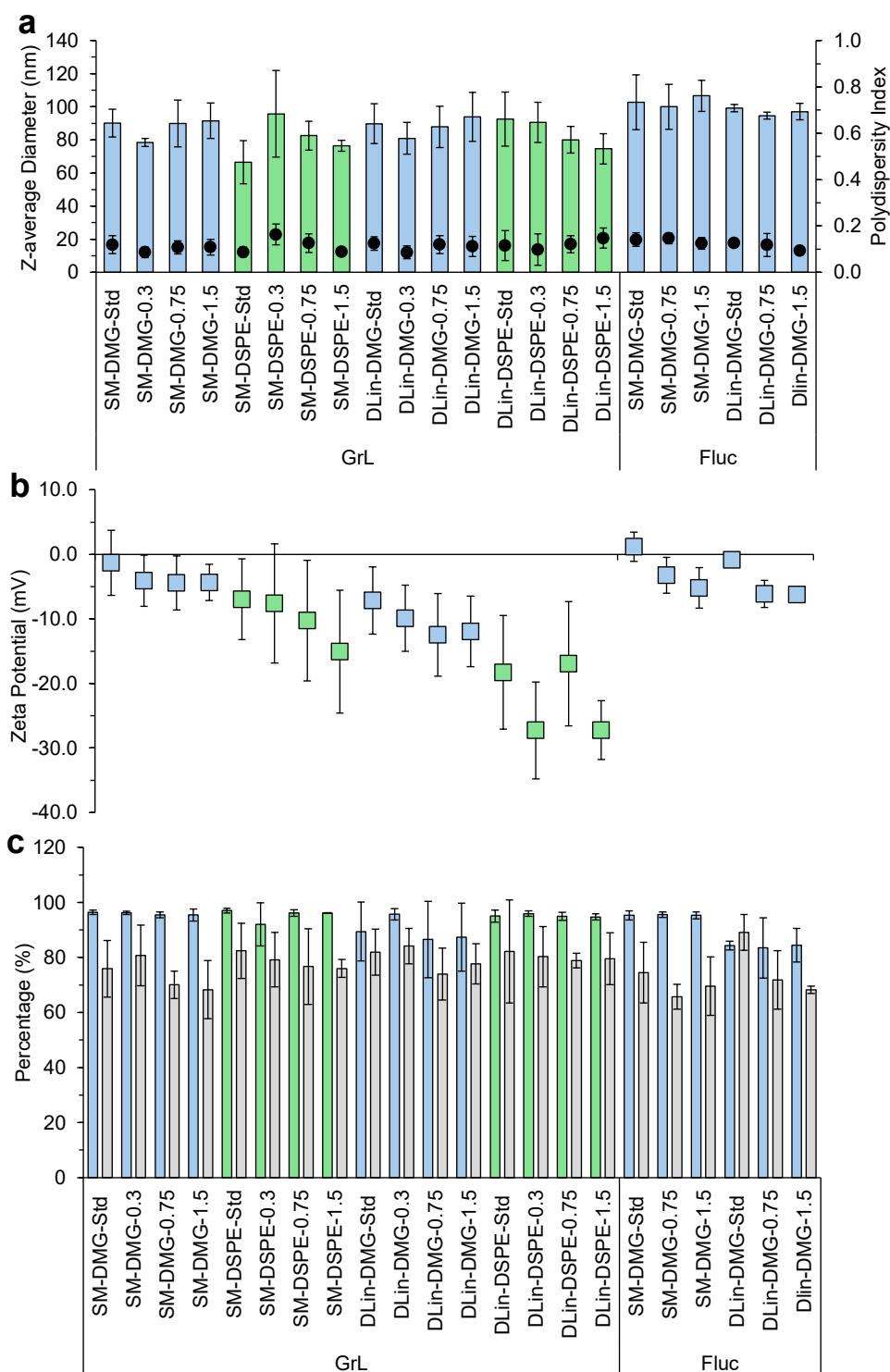


Figure 5-5 Critical Quality Attribute Testing of LNPs with Varied Surface Modification Levels. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:1.3:0.3% or 10.9:42:45.5:0.8:0.8% or 10.9:42:45.5:1.6%) encapsulating Green Lantern mRNA or Fluc mRNA at a final concentration of 0.04 mg/mL. (a) represents z-average diameter (nm) (bars) and polydispersity index (circles) while (b) represents the zeta potential (mV) and (c) represents the encapsulation efficiency (%) (coloured bars) and mass balance (grey bars). Results are representative of the mean $\pm$ standard deviation (n=3-12).

Figure 5-5a shows the LNP sizes and polydispersity index for LNPs manufactured with varying surface-targeting levels and different LNP compositions. Regardless of formulation, GrL-LNPs range between 66-94 nm with PDI consistently <0.15 (Figure 5-5a). The same can be said for Fluc-LNPs; however, these were generally larger than GrL-LNPs, with sizes ranging from 97-108 nm and PDI again consistently <0.15 (Figure 5-5a). There were no significant differences in size or polydispersity between LNPs with the same ionizable lipid and payload but different surface-targeting levels ($p>0.05$). For GrL-LNPs, the slight negative shift of zeta potential generally increases as the surface targeted modification levels increase, excluding DLin-DSPE-LNPs, which exhibited more variable results (Figure 5-5b). Overall, zeta potentials range between -2 and -28 mV. The same negative shift is evident for Fluc-LNPs, but to a much lesser extent, ranging from -7.5 to -1.5 mV (Figure 5-5b). However, the negative shift relative to standard formulations is not statistically significant ($p>0.05$). In both GrL-LNPs and Fluc-LNPs, there was a slight decrease in encapsulation efficiency for DLin-LNPs; however, no changes were significant ($p>0.05$) and ranged between 83-97% (Figure 5-5c). Furthermore, mass balance was unaffected by formulation variation ranging between 68-85% (Figure 5-5c).

5.4.4 Investigating the Effect of Increasing Surface Modification on *In vitro* Protein Expression

It was subsequently deemed necessary to understand how altering the surface-modification percentages could affect targeted delivery. To investigate this, GrL-LNPs recorded in section 5.4.3 were used to analyse GFP expression and cell viability in both RAW264.7 cells and polarised RAW264.7 cells. The quantified GFP expression and cell viability are shown in Figure 5-6.

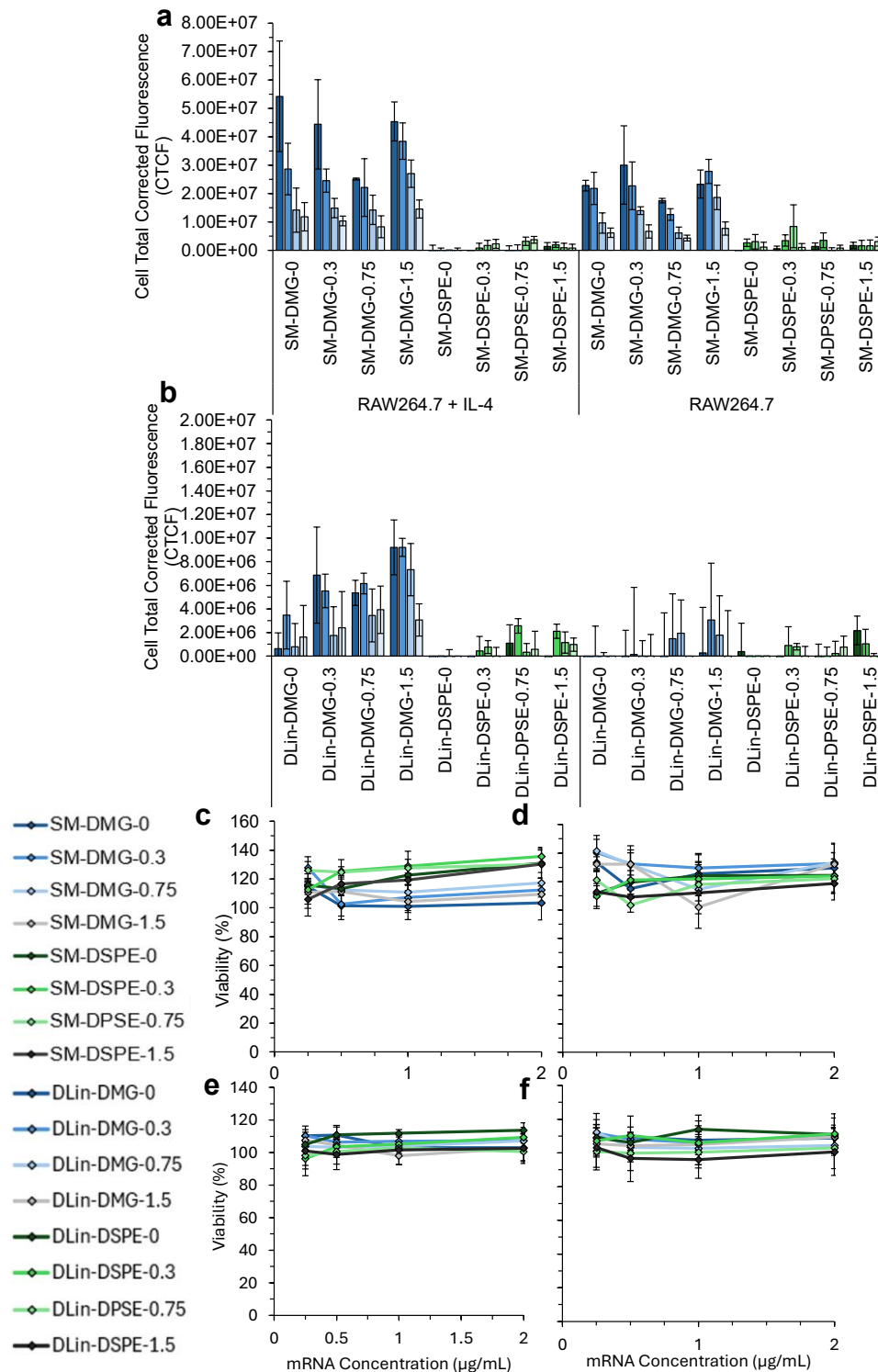


Figure 5-6 In Vitro GFP Expression of LNPs with Varied Surface Modification Levels. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:1.3:0.3% or 10.9:42:45.5:0.8:0.8% or 10.9:42:45.5:1.6%) encapsulating Green Lantern mRNA at a final concentration of 0.04 mg/mL. (a) represents the GFP expression of varied SM-102-LNPs following treatment for 24 hours while (b) represents the GFP expression of DLin-MC3-DMA-LPs following treatment for 24 hours. Cell viability was monitored in RAW264.7 cells polarised with IL-4 for both SM-102 (c) and DLin-LNPs (e) as well as RAW264.7 with no polarisation for SM-102 (d) and DLin-LNPs (f). Results are representative of the mean $\pm$ standard error of the mean (n=3).

Figure 5-6a-b shows GFP expression across all LNP formulations, and overall, it can be seen that for LNPs containing DMG-PEGs, SM-102-LNPs express higher than DLin-LNPs. Furthermore, across the entire dataset, there were no significant differences ($p>0.05$) between formulations used to treat polarised and non-polarised macrophages. However, expression results were formulation dependent.

Figure 5-6a highlights the SM-102 expression in both polarised and unpolarised RAW264.7 cells. All DMG-LNPs exhibited non-significant increases in expression in the polarised subset compared to non-polarised ($p>0.05$). The largest fold change increases in expression were 2.4 and 2.3, and were experienced at 2 $\mu\text{g/mL}$ for SM-LNPs and 0.5 $\mu\text{g/mL}$ for SM0.75-LNPs, respectively (Figure 6a), with the remaining formulations all exhibiting a 1.1 to 1.9 fold increase in polarised cells compared to non-polarised cells (Figure 5-6a). On the other hand, Figure 5-6b represents the same cell conditions but treated with DLin-based LNPs. Similarly to SM-102-LNPs, no significant differences were seen with DLin-LNPs, but the highest fold change increase was seen at a treatment concentration of 1 $\mu\text{g/mL}$ for the DMG0.3-LNP (Figure 5-6b). Remaining DMG-LNPs exhibited between 0.3 – 10 fold change increases. For both experimental mental groups, DSPE-LNPs did not express well and showed no efficacy differences between polarised and non-polarised cells.

Finally, Figure 5-6c/d represents cell viability following treatment of SM-LNPs in polarised and non-polarised cells, respectively. Figure 5-6e-f shows the cell viability following treatment with DLin-LNPs in polarised and non-polarised cells, respectively. Overall, no significant changes in cell viability were observed, indicating that the targeting is not toxic ($p>0.05$).

5.4.5 Confirmation of Target Selectivity

While we were able to highlight that expression can be increased in polarised cells, we lack the evidence to suggest that this is due to the selective binding and

uptake of LNPs via cells that are positive for the ligand target – the CD206 receptor. In an attempt to expand our knowledge, we selected size formulations to perform flow cytometry analysis, in which we will monitor GFP and CD206 expression in polarised and non-polarised cells following treatment with LNPs. The results of the analysis are shown in Figure 5-7. Due to poor expression seen in previous results, DSPE-LNPs were excluded from the following analysis.

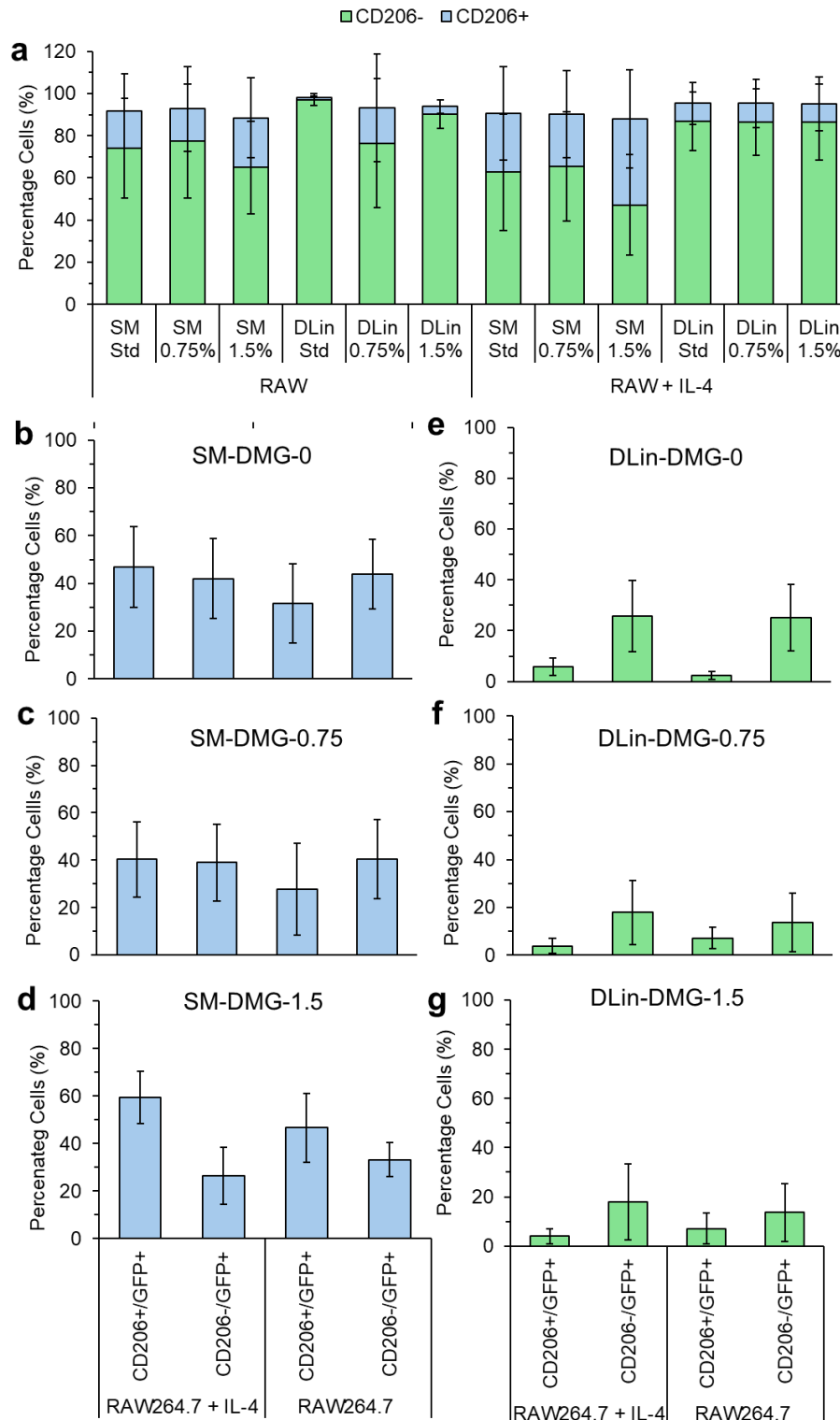


Figure 5-7 Confirming Targeting Selectivity of LNPs. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:0.8:0.8% or 10.9:42:45.5:1.6%) encapsulating Green Lantern mRNA at a final concentration of 0.04 mg/mL. LNPs were used to treat cells which were subsequently analysed using flow cytometry to identify (a) the percentage of cells in each sample that were CD206+ or CD206- while the quantification of GFP signal in CD206- and CD206+ cells are shown for SM-102-LNPs (b), SM-102-0.8%-LNPs (b), SM-102-1.6%-LNPs (c), DLin-LNPs (d), DLin-0.8%-LNPs and (e) DLin-1.6%-LNPs. Analysis was conducted in both polarised and non-polarised RAW264.7 cells. Results are representative of the mean $\pm$ standard error of the mean (n=3).

Figure 5-7a represents the percentage of cells in the tested population that are CD206+ (blue) and CD206- (green). Contrary to the literature, which assumes that non-polarised RAW264.7 cells do not express the CD206 receptor, our results indicate that, in all tested samples, the CD206 receptor is present in both polarised and non-polarised RAW264.7 cells (Figure 5-7a). For SM-102-, SM-102-1.6%- and DLin-1.6%-LNPs no significant differences were seen between CD206-/+ percentages ($p>0.05$). However, for SM-102-0.8%-, DLin-0.8%-, and DLin-LNPs, there were significantly more ($p<0.01$) CD206- cells compared to CD206+ cells in the non-polarised RAW264.7 cells.

Figure 5-7b-f shows the percentage of cells that are CD206+ and GFP+ compared to CD206- and GFP+ for both polarised and non-polarised RAW264.7 cells for all treatment LNPs. Initially, it can be seen that DLin-LNPs exhibited lower expression than SM-102-LNPs, which agrees with what we previously saw in results section 5.3.4. SM-102-LNPs consistently exhibited >68% transfection compared to DLin-LNPs, which exhibited only 20-38% transfection (Figure 5-7b-f). In addition, all tested LNPs showed either greater expression in CD206- cells or only minor changes in expression between CD206- and CD206+ populations, except for SM-102-1.6%-LNPs, which exhibited non-significantly increased expression in CD206+ cells (Figure 5-7d). SM-102-1.6%-LNPs showed a 33% increase in CD206+/GFP+ in polarised RAW264.7 cells and a 13% increase in CD206+/GFP+ in non-polarised RAW264.7 cells (Figure 5-7d).

5.4.6 Monitoring *In vivo* Expression

In previous sections, we have demonstrated that targeted LNPs increase protein expression without adverse effects. To further understand the targeted formulations, the *in vivo* protein expression of the target and control LNPs were monitored for 3 weeks in BALB/c mice following I.V. injection. In addition, mice were subjected to a micro-CT scan 6 hours post-injection to monitor protein

expression. The results of the time-course study are shown in Figure 5-8, while the micro-CT scans are shown in Figure 5-9.

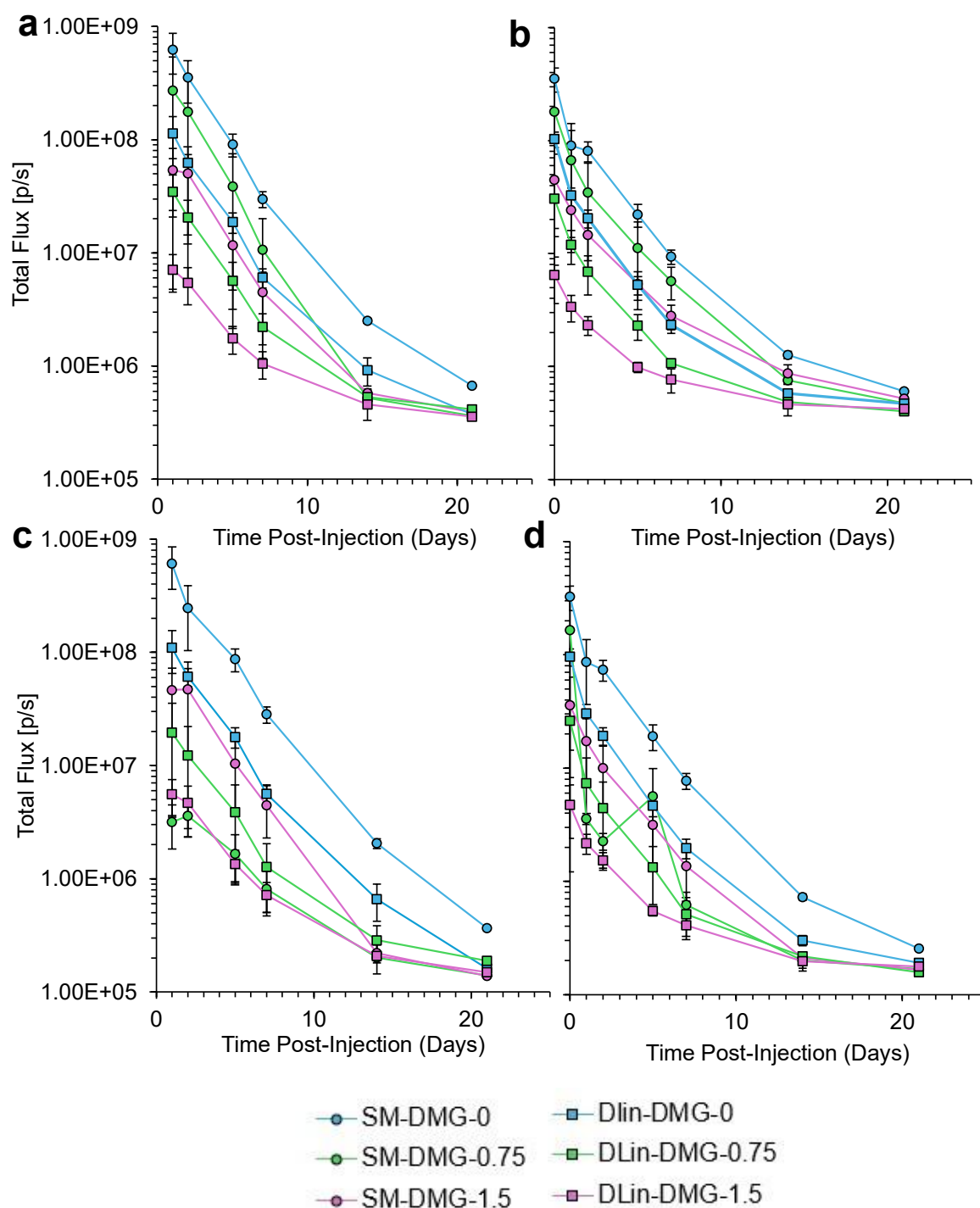


Figure 5-8 Monitoring In Vivo Protein Expression and Biodistribution of LNPs. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:0.8:0.8% or 10.9:42:45.5:1.6%) encapsulating Fluc mRNA at a final concentration of 0.04 mg/mL. LNPs were used to treat cells BALB/c mice by intravenous injection of 1 μ g mRNA in the tail vein. Bioluminescence images were captured on the IVIS Spectrum at 6, 24 and 48 hours as well as Day 5, 7, 14, and 21 post injection and signals were quantified on Living Image v4.0. The quantified bioluminescence signals of LNPs were monitored in both the whole mouse in both ventral (a) and dorsal positions (b) alongside monitoring liver expression in the ventral (c) and dorsal (d) positions. Results are representative of the mean $\pm$ standard error of the mean (n=3) with Y-axis presented in Log form.

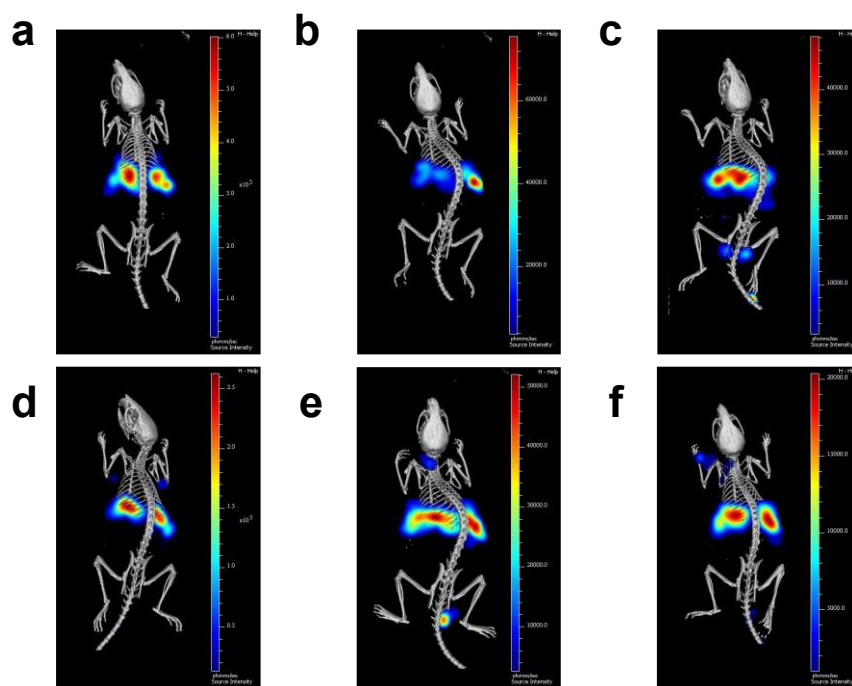


Figure 5-9 Micro-CT Scans of BALB/c mice 6 hours post-injection with control or targeted LNPs at a concentration of 1 $\mu\text{g/mL}$. Images were conducted using the Quantum GX2 Micro-CT and are representative of SM-102-LNPs (a), SM-102-0.8%-LNPs (b), SM-102-1.6%-LNPs (c), DLin-LNPs (d), DLin-0.8%-LNPs (e) and DLin-1.6%-LNPs. Scales are representative of photons/sec intensity and scale maximum, and minimums are (a) $1.0 \times 10^5 - 6.0 \times 10^5$, (b) $2.0 \times 10^4 - 6.0 \times 10^4$, (c) $1.0 \times 10^4 - 4.0 \times 10^4$, (d) $0.5 \times 10^5 - 2.5 \times 10^5$, (e) $1.0 \times 10^4 - 5.0 \times 10^4$ and (f) $5.0 \times 10^3 - 2.0 \times 10^4$. Results are representative of one repeat; imaging was carried out at an $n=3$.

Figure 5-8a-b represent the whole mouse bioluminescence signals in either dorsal or ventral imaging positions. Both imaging positions show similar expression patterns and levels, with expression decreasing as the targeting surface modification increases; this holds for both SM-102>SM-102 0.8%>SM-102 1.6% and DLin>DLin 0.8%>DLin 1.6% in both positions. On the other hand, Figure 5-8c-d represent the liver bioluminescence signals in the dorsal or ventral positions. These show the same patterns as whole-body expression, with bioluminescence decreasing from SM-102>SM-102 0.8%>SM-102 1.6% and DLin>DLin 0.8%>DLin 1.6%.

Figure 5-9 provides a visual representation of the distribution of protein expression in BALB/c mice following treatment with LNPs. It can be observed that

all formulations have similar biodistribution, with high expression in the liver (Figure 5-9). This is further emphasised in Figure 5-8, where we have quantified bioluminescence signals over a 3-week period in both whole mouse (a/b) and liver (c/d) regions of interest.

5.4.7 Evaluating Targeting Specificity *In vivo*

M2 macrophages, which possess the CD206 receptor, are generally activated in response to cellular signals. We aimed to determine whether we could establish any targeting specificity *in vivo* using only baseline levels of M2 macrophages. To evaluate this, we treated BALB/c mice with control and targeted LNPs via I.V. administration in the tail vein. 24 hours post-injection, mice were culled, and the heart, kidney, lungs, liver and spleen were extracted for ex vivo imaging to visualise GFP expression. The results are shown in Figures 5-10. Furthermore, liver and spleen cells were analysed by flow cytometry to identify CD206+ cells and assess their expression levels. The results are shown in Figure 5-11 and Table 5-1/5-2.

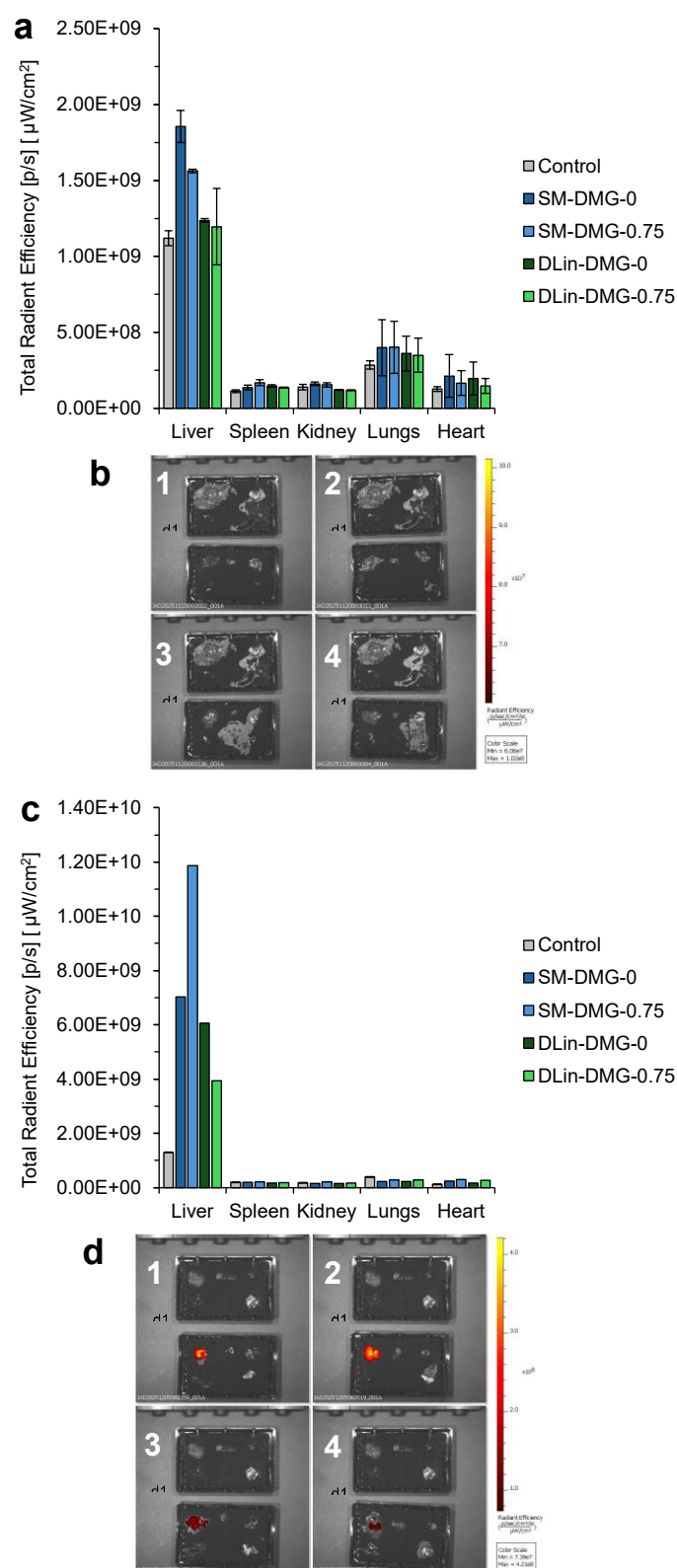


Figure 5-10 Ex Vivo Evaluation of GFP Expression 24 hours post-injection with control or targeted LNPs. Images were conducted using the IVIS Spectrum and are representative of SM-102-LNPs, SM-102-0.8%-LNPs DLin-LNPs and DLin-0.8%-LNPs. Protocols were initially carried out by treating with $1 \mu\text{g}$ mRNA (a) and (b), and later followed by increased concentrations of $7 \mu\text{g}$ (c) and (d). (a) and (c) represent the quantified GFP signal from each organ, while (b) and (d) show the representative images with SM-102-LNPs (b1/d1) (top left), SM-102-0.8%-LNPs (b2/d2) (top right), DLin-LNPs (b3/d3) (bottom left) and DLin-0.8%-LNPs (b4/d4) (bottom right). In (b) images layout is liver (top left), spleen (bottom left), kidney (centre top), lungs (top right) and heart (bottom right) whereas in (d) images layout is liver (top left), spleen (bottom left), kidney (centre top), heart (top right) and lungs (bottom right). Scales are representative of radiant efficiency and scale maximum, and minimums are (b) 6.06×10^7 to 1.02×10^8 and (d) 7.39×10^7 to 4.21×10^8 . Results are representative of mean $\pm$ standard deviation (a(n=2)) and (c(n=1)).

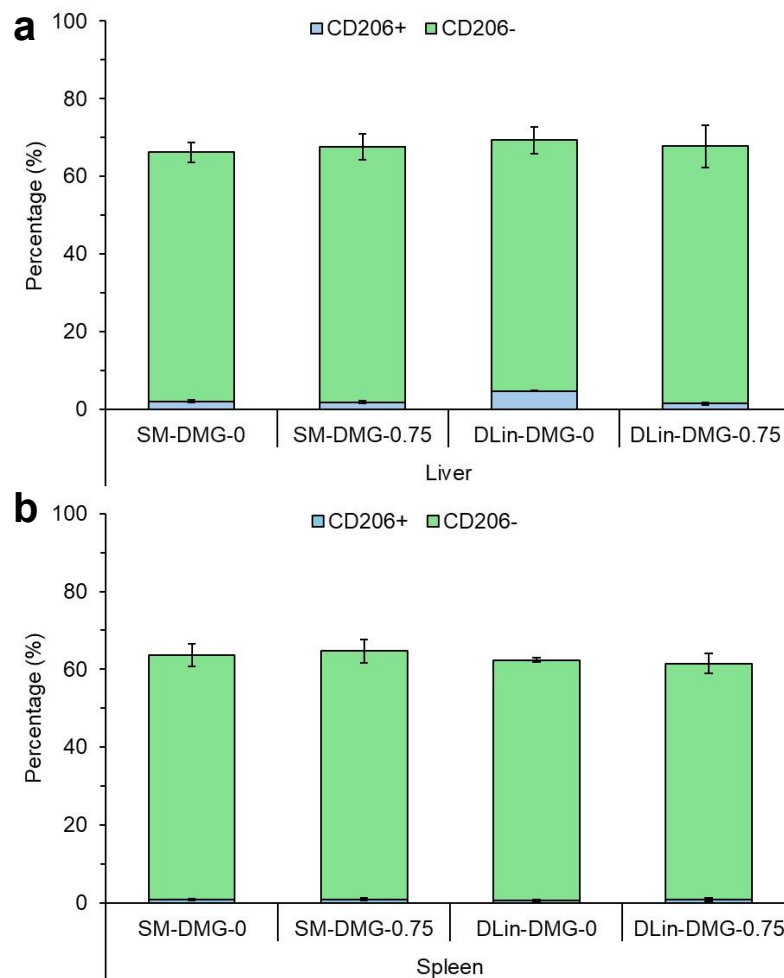


Figure 5-11 Evaluating Targeting Selectivity of LNPs In Vivo. LNPs consisted of DSPC:cholesterol:SM-102:DMG or DSPE-PEG₂₀₀₀ (10.9:42:45.5:1.6%) +/- DMG or DSPE-PEG₂₀₀₀-Azide (10.9:42:45.5:0.8:0.8%) encapsulating Green Lantern mRNA. LNPs were used to treat BALB/c mice at either 1 µg/mL or 7 µg/mL via I.V. injection and 24 hours post injection mice were culled and liver and spleen excised for flow cytometry analysis. (a) the percentage of liver cells in each sample that were CD206+ or CD206- while (b) represents the percentage of spleen cells in each sample that were CD206+ or CD206-. Results are representative of the mean ± standard deviation (n=3).

Table 5-1 Quantification of GFP signals and CD206 levels from Liver and Spleen extracted from BALB/c mice following 24-hour treatment with 1 µg/mL mRNA-LNPs quantified using flow cytometry. Results are representative of the mean ± standard deviation (n=2).

	Liver		Spleen	
	CD206+/GFP+	CD206-/GFP+	CD206+/GFP+	CD206-/GFP+
SM-102	0.2 ± 0.2	1.7 ± 1.1	0.4 ± 0.1	0.4 ± 0.2
SM-102 0.8%	0.2 ± 0.1	1.6 ± 0.5	0.3 ± 0.1	0.3 ± 0.3
DLin	0.4 ± 0.2	4.2 ± 3.0	0.2 ± 0.1	0.4 ± 0.1
DLin 0.8%	0.1 ± 0.1	1.2 ± 0.7	0.3 ± 0.2	0.5 ± 0.2

Table 5-2 Quantification of GFP signals and CD206 levels from Liver and Spleen extracted from BALB/c mice following 24-hour treatment with 7 µg/mL mRNA-LNPs quantified using flow cytometry. Results are representative of the mean ± standard deviation (n=1).

	Liver		Spleen	
	CD206+/GFP+	CD206-/GFP+	CD206+/GFP+	CD206-/GFP+
SM-102	5.7 ± 4.2	2.6 ± 3.8	34.4 ± 6.9	0.1 ± 0.1
SM-102 0.8%	7.0 ± 4.2	1.9 ± 2.3	32.7 ± 2.9	0.2 ± 0.2
DLin	4.3 ± 1.0	2.5 ± 4.2	13.6 ± 9.6	0.1 ± 0.1
DLin 0.8%	6.5 ± 5.2	0.7 ± 1.2	13.1 ± 12.6	0.1 ± 0.2

Initially, Figure 5-10 represents the GFP expression in ex vivo organs imaged on the IVIS. We can see that quantified signals from lower treatment concentrations (1 µg/mRNA) are minimal and cannot be picked up by the IVIS (Figure 5-10a/b). However, when treatment concentrations were increased (7 µg/mRNA), expression was visibly evident in the liver of all samples (Figure 5-10b/d). Furthermore, when signals were quantified, liver expression was increased in SM-102-0.8%-LNPs – a fact which was not evident in biodistribution studies (Figure 5-9c/d). Because they are known as macrophage-rich organs, the liver and spleen were selected for Flow Cytometry analysis, with results shown in Figure 5-11 and Tables 5-1 and 5-2.

Figure 5-11 highlights the percentage of cells that were positive for the CD206 receptor in Liver (a) and spleen (b) extracts. Overall, the liver showed increased CD206+ cells compared to the spleen, but overall CD206+ levels were low, with percentages between 0.5-5% (Figure 5-11). From Table 5-1, it is evident that low concentrations of GFP-LNPs were not sufficient to produce high GFP expression levels; this agrees with data from quantified ex vivo imaging in Figure 15-0a. On the contrary, when GFP-LNP treatment concentration was increased (Table 5-2), increases in GFP expression were seen specifically in the spleen. However, due to the low levels of CD206+ cells in the population, it is unclear how significant this percentage is. Additionally, SM-102-LNPs show higher expression than DLin-LNPs, which is consistent with data from *in vitro* and *in vivo* testing (Table 5-2).

5.5 Discussion

The therapeutic landscape of LNPs is shifting from standard formulation optimisation to engineering formulations capable of selectively targeting. With the aim of enhancing knowledge in this area, we developed a novel M2 macrophage-targeted LNP by conjugating a DBCO-trisFucose ligand to the surface of several LNP formulations. We then demonstrated enhanced *in vitro* protein expression with no evident shift *in vivo* biodistribution.

Initial research focused on optimisation of binding conditions for the DBCO-trisfucose ligand. The SPAAC reaction that occurs between DBCO and azide has been recorded as having second-order rates of 0.01-60 M⁻¹S⁻¹, with the fastest reactions usually seen at room temperature and occasionally with the addition of a solvent to minimise the lipophilicity of DBCO [227]. However, during our study, we aimed to conjugate our DBCO-trisFucose to our LNPs post-manufacture, meaning the LNPs would be in an aqueous pH 7.5 environment, in which adding organic solvents would not be ideal due to the risk of LNP aggregation and breakdown. Previous research has shown that SPAAC reaction rates are highly tolerable in a range of neutral buffers [228]. Therefore, we opted to complete the reaction in the post-purification 10 mM Tris buffer, pH 7.5, at either 4 °C or room temperature. Furthermore, due to varied second-order rate reactions, conjugation was carried out for 24 hours and at an excess ratio of (DBCO:Azide). LNPs were then either left unpurified or purified again to remove any excess DBCO-trisFucose, and the CQAs of the LNPs were monitored (Figure 5-3a-c, Figure 5-4a-c, and Figure 5-5). In general, no significant changes in size, PDI, or payload encapsulation between the control and targeted formulations. Wang et al. reported minimal changes in size when they manufactured liposomes containing a mannose-cholesterol conjugate synthesised via a click reaction [229]. Additionally, Goswami et al. demonstrated that increasing the molar percentage incorporation of a mannose-cholesterol amine conjugate into DLin-

LNPs had no significant effect on size and PDI [230]. Finally, Goswami et al. later demonstrated that incorporating mannans of different lengths had minimal effects on LNPs size, PDI, and encapsulation efficiency [231]. This overall enhanced our confidence that the inclusion of our targeted ligand did not influence the LNP structure. It is important to mention that the zeta potential of LNPs did become more negative as surface modification levels were increased (Figure 5-5b). The control LNPs exhibit the classic near-neutral surface potential; however, as the ratio of DBCO-trisFucose is incorporated, a systematic shift towards negative values is noted, indicating that the ligand can offer a degree of tunability in terms of LNP surface properties. In general, cationic and neutral LNPs are thought to be more beneficial due to enhanced uptake into endosomal escape [232]; however, anionic lipids have recently been shown to drive uptake in macrophages [233].

We next established the relationship between targeting and *in vitro* protein expression using RAW264.7 cells that were either polarised by IL-4 or non-polarised (Figure 5-6). Our overall trends consistently indicate that SM-102-LNPs showed enhanced protein expression compared to DLin-LNPs. This has previously been reported by Lindsay et al., who conducted a systematic review of major commercially available LNP formulations across several cell lines and *in vivo* [234]. This gave us confidence that incorporating DBCO-trisFucose did not negatively affect LNP efficacy. While we were able to show that inclusion of the DBCO-trisFucose did increase *in vitro* protein expression for both SM-102-DMG-LNPs and DLin-DMG-LNPs, *in vitro* protein expression of the control LNP was also enhanced in polarised macrophages (M2) compared to non-polarised (M0) macrophages. This can be theorised to result from M2 cells having higher phagocytic activity. Initial research demonstrated that M2 macrophages employ continuous micropinocytosis and that proteolytic levels in macrophages increase with IL-4 addition [235]. Furthermore, initial *in vitro* optimisation showed that SM-102-LNP expression is high in HEK-293 cells (Figure 5-4d), indicating the

formulation's high efficacy prior to targeting modification. Due to their low initial expression, DLin-LNPs were more indicative of increased protein expression with increasing surface modification levels. While this was promising, flow cytometry was used to determine whether increased expression was associated with higher CD206 expression. We demonstrated that polarisation led to varied levels of CD206 expression, and only SM-102 LNPs with 100% DBCO-trisFucose surface modification achieved CD206+-specific delivery. This generally agrees with Figures 5-6 and highlights that 100% surface modification is required to ensure that the ligand is not shielded and is fully accessible to the CD206 receptor. We also identified that non-polarised M0 expressed the CD206 receptor, which explains why targeted formulations showed baseline expression in the non-polarised *in vitro* model.

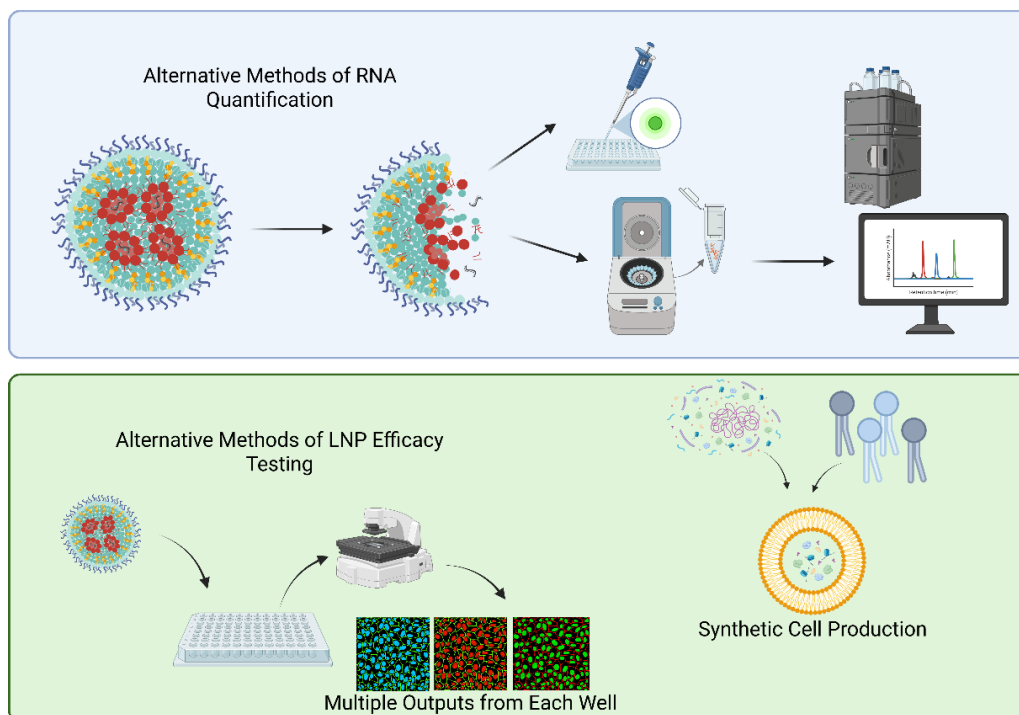
Finally, we monitored *in vivo* expression and biodistribution, and we found that incorporation of our DBCO-trisFucose had no significant effect on protein expression over a three-week period. After establishing that the expression wasn't limited under general circumstances, we decided to perform *ex vivo* analysis to determine whether targeting improved delivery in CD206+ cells. Overall, we showed that GFP signals could not be imaged on the IVIS at lower treatment concentrations and only became visible at higher concentrations. *Ex vivo* imaging of the organs revealed that only the liver showed increased expression with the SM-102-0.8% targeted formulation; however, both the liver and spleen were selected for further analysis. We demonstrated that overall CD206+ levels in the spleen were low, whereas they were slightly increased in the liver. It has previously been reported that Kupffer cells in the liver can be differentiated into KC1 and KC2 subtypes, comprising 85% and 15% of the population, respectively, with KC2 expressing the CD206 receptor [236]. We reported 0.6-5% CD206+ cells, which is below the standard, and observed low GFP-positive signals in flow cytometry, which is inconsistent with the IVIS® images. It is possible that impurities in collagenase enzymes used to break down

the liver could degrade GFP; however, GFP expression has previously been shown to increase in murine tissues with collagenase and hyaluronidase present [237]. Regardless, GFP expression was monitored in the spleen, but CD206+ levels were so low that the response is most likely negligible. We believe additional studies in mice in which M2 macrophages are stimulated could yield enhanced *in vivo* targeting data.

5.6 Conclusions

In conclusion, we established a strong foundation for macrophage-targeted LNPs by developing a novel DBCO-trisFucose linker for incorporation into LNPs, enabling formulation variation. We demonstrated receptor-selective targeting, resulting in increased protein expression *in vitro*. Furthermore, targeted LNPs demonstrate consistent, similar *in vivo* expression to non-targeted LNPs, indicating that formulation modifications do not compromise *in vivo* efficacy, but notable targeting was difficult to demonstrate in our current models. Overall, this work provides a robust framework that can be further used to enhance M2-specific delivery of LNPs.

Chapter 6: Breaking the Bubble: Alternative Methods to RNA-LNP Quantification and Efficacy Testing



Type of Publication	☐ Published article ☐ Accepted for publication ☐ Submitted for publication ☒ Part-Drafted for publication/application notes ☐ Not applicable
Citation Details (only applicable if published)	N/A
Chapter Inclusion Details	☐ This chapter is a word-for-word reproduction of the published article ☒ This chapter contains minor revision of a drafted application note for RiboDyEE™ ☐ This majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	Sarah Lindsay and Rebecca Macfarlane assisted with RiboDyEE™ user testing. RiboDyEE™ was developed by Tocris Team in BioTechne.

6.1 Introduction

RNA-LNPs are expected to dominate the future therapeutic landscape, following the success of the COVID-19 vaccines [238]. With many papers starting to highlight the need for increased stability and innovation beyond LNP technologies [239]. However, as more products are introduced to the market, the level of orthogonal analysis required to pass quality control will increase.

While key physicochemical characterisations of LNPs, including particle size, polydispersity index, and zeta potential, are widely regarded as critical determinants of efficacy, the analytical methods used to measure these parameters vary and each introduces inherent biases. Parot et al. conducted a study focused on using different analytical methods to determine how they link to the safety and efficacy of RNA-LNPs, where they showed that routine quality control measurements are minimal, and researchers should strive for true in depth analysis [125]. Nevertheless, the central functional component of these systems is the mRNA payload itself, and accurate quantification of the RNA dose within the formulation is essential to ensure both reproducibility and meaningful interpretation of biological outcomes.

The method employed to analyse RNA payloads is highly dependent upon what attribute is being quality tested. Orthogonal techniques are often employed to ensure regulatory compliance through sequence and structural validation [240]. However, to specifically determine RNA encapsulation within LNPs, a fluorescence-based assay, commonly the Ribogreen™ assay, is employed.

While the Ribogreen™ assay is highly considered to be the gold standard, it does have limitations, including user-to-user variability and a lack of ability to distinguish intact RNA from degraded RNA [241]. Researchers often have to employ numerous techniques to help monitor mRNA integrity and size, such as capillary gel electrophoresis and/or ion-pair reversed-phase liquid chromatography (IP-HPLC) [242].

Furthermore, the dyes employed in these assays are not inherently specific to LNP encapsulation, emphasising the need for careful optimisation of assay conditions prior to implementation. A key aspect of this optimisation is the inclusion of a detergent to disrupt the LNP structure and enable release of the encapsulated mRNA. The leading detergent of choice is now Triton X-100; however, there is an increasing need to replace this due to its potential human and environmental impact [160]. In this context, Schultz et al., demonstrated that Tween 20 can serve as a suitable alternative [136].

While the Ribogreen™ assay serves as a functional assay to confirm the presence of the RNA payload, it cannot verify biological efficacy. Both the European Pharmacopoeia and the European Medicines Agency (EMA) offered guidelines which emphasise biological testing through producers, such as *in vitro* cell-based assays [243].

While 2D cell culture models are the standard method for *in vitro* testing, LNP formulation screening has shown a large variation in efficacy results across different cell lines and following different storage conditions [234, 243]. Highlighting the importance of selecting the correct testing model, Muzard et al. conducted a review article stating how 2D monolayers lack any complex biological environment and heterogeneity [244]. Therefore, enhanced *in vitro* testing models, such as 3D spheroids, are gaining popularity, specifically in the area of cancer research [245]. In addition, synthetic cell models are becoming more prominent, offering the advantage of lower costs and lack of contamination risk while still being able to mimic biological behaviour and function [246].

This chapter aimed to enhance RNA quantification and *in vitro* efficacy assessment methods for LNP formulations in order to improve overall quality control. To achieve this, a fluorescent RNA detection assay was first optimised by identifying appropriate control payloads and evaluating emerging dyes as potential cost-effective alternatives. An HPLC-PDA method was then developed

and validated to enable robust quantification of RNA in LNPs, providing an orthogonal technique to support assay reliability. Finally, *in vitro* evaluation strategies were advanced by developing a combined multi-output cell assay and a synthetic cell lysate testing model, enabling improved assessment of LNP performance.

6.2 Materials and Methods

6.2.1 Materials

Polyadenylic acid (Poly A), Triton X-100, cholesterol, Triethylammonium Acetate, HPLC-grade methanol, HPLC-grade acetonitrile, propidium iodide, 1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiIC), glucose, potassium acetate, ammonium acetate, magnesium acetate, PEG6000, mineral oil and the amino acid kit were purchased from Sigma (Merck Life Science UK Ltd., Gillingham, UK). The Quanti-iT™ Ribogreen™ Assay Kit, Tris-EDTA (TE) buffer, sodium citrate Buffer, Tris-HCL buffer, NucBlue™ LiveReadyProbes™ Reagent (Hoechst 33342), Pierce™ IP Lysis buffer and HEPES buffer were all purchased from Thermo Fisher Scientific (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The cell culture media, including Phenol Red Free Dulbecco's Minimal Essential Media (PR Free DMEM), Dulbecco's Minimal Essential Media (DMEM), sodium pyruvate, penicillin, streptomycin and TrypLE, were all purchased from Gibco Technologies. The ionizable lipids Heptadecan-9-yl 8-((2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino) octanoate (SM-102) and (6Z, 9Z, 28Z, 31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (D-Lin-MC3-DMA) alongside 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and RiboDyEE™ reagent were acquired from Tocris (Bristol, UK). The remaining lipids, including 6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium (ALC-0315) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), were acquired from Avanti Polar Lipids (Alabaster, AL,

USA). HPLC-grade ethanol was supplied by Supelco (Bellefonte, PA, USA). The EZ Cap™ Firefly Luciferase mRNA (5-moUTP) was acquired from APExBIO (Houston, TX, USA), while CPI (Centre for Process Innovation, Sedgefield, UK) supplied the Green Lantern mRNA and Fluc-GrL Fusion mRNA. Finally, HEK-293 cells (CRL-1573) and A549 cells (CRM-CCL-185) were purchased from American Type Culture Collection (ATCC).

6.2.2 Calibration Curve Optimisation

Either Poly A or the Quanti-iT™ Ribogreen™ Assay rRNA standard was used to prepare stock solutions at 4 µg/mL and 0.4 µg/mL, respectively, which were subsequently used to generate the calibration curves as shown in Table 6-1 and 6-2. Calibration curves were generated over six days to assess interday variability, while intraday variability was evaluated by measuring the same plate three times in one day. Prior to measurement, Ribogreen™ dye was added to the high (Table 6-1) and low (Table 6-2) curves using either a 200X or 500X solution, respectively. Plates were then quantified on both the POLARstar OMEGA Microplate reader (BMG LABTECH, Ortenberg, Germany) at an excitation of 475 nm and emission of 550 nm or on the GloMax® Discover Microplate Reader (Promega, Chilworth, UK) with an excitation of 475 nm and emission of 500-550 nm.

Table 6-1 Volumes required to generate a high calibration curve ranging from 0 to 1000 ng/mL for use in the Ribogreen™ Assay.

Volume of Stock (4 µg/mL) (µL)	Volume of 2%-Triton 1X TE (µL)	Volume of 1X TE (µL)
50	50	0
40		10
30		20
20		30
10		40
5		45
2.5		47.5
0		50

Table 6-2 Volumes required to generate a low calibration curve ranging from 0 to 200 ng/mL for use in the Ribogreen™ Assay.

Volume of Stock (0.4 µg/mL) (µL)	Volume of 1X TE (µL)
100	0
75	25
50	50
25	75
12.5	87.5
5	95
2.5	97.5
0	100

6.2.3 Ribogreen™ vs RiboDyEE™

6.2.3.1 LNP Manufacture

LNPs lipid phase consisted of either DSPC, cholesterol, SM-102 or DLin-MC3-DMA and DMG-PEG2000 at a ratio of 10:38.5:50:1.5% or DSPC, cholesterol, ALC-0315 and ALC-0159 at a ratio of 9.4:42.7:46.3:1.6% in absolute ethanol (>99.5%). The aqueous phase consisted of either poly (A) or Firefly Luciferase (Fluc) mRNA in 50 mM citrate buffer (pH 5). LNPs were manufactured on Nova™ IJM Benchtop (Helix Biotech, TN, USA) at a flow rate ratio of 3:1 (aqueous:lipid) and a total flow rate of 15 mL/min. LNPs were purified using size-exclusion chromatography in 10 mM Tris, pH 7.5, before analysis.

6.2.3.2 Assay Protocol

Poly A or Firefly Luciferase mRNA (Fluc) was used to prepare stock solutions of either 4 µg/mL or 0.2 µg/mL and used to generate the calibration curves shown in Table 6-3 and 6-4, respectively. RNA-LNP samples should be diluted with 1X TE buffer to a final volume of 350 µL at a concentration of 3 µg/mL RNA. For plating the LNPs, six wells (volume of 50 µL) of each RNA-LNP sample should be added to the appropriate wells on the plate as shown below. For each RNA-LNP sample, 50 µL 1X TE buffer should be added to three of the wells, while 50 µL of 2%-Triton

TE buffer is added to the remaining three wells (final volume per well will be 100 μ L). A blank should also be prepared using the same dilution as the RNA-LNP sample, substituting the buffer for the LNP sample. Interday analysis of curves is performed using three curves generated on three separate days, while intraday analysis is the measurement of three separate curves prepared on one day. Fluorescence plate readouts were conducted using the GloMax® Discover Microplate Reader (Promega, Chilworth, UK) using emission filter 500-550 nm and excitation filter Blue 475 nm. Both dyes were diluted in 1X TE buffer to achieve a 200X solution used in wells with 2%-Triton TE buffer and a 500X solution used in wells with 1X TE buffer, 100 μ L of reagent required per well.

Table 6-3 Volumes required to generate a high calibration curve ranging from 0 to 1000 ng/mL for use in the Ribogreen™ vs RiboDyEE™ Assay.

Volume of Stock (4 μ g/mL) (μ L)	Volume of 2%-Triton 1X TE (μ L)	Volume of 1X TE (μ L)
50	50	0
40		10
30		20
20		30
10		40
5		45
2.5		47.5
0		50

Table 6-4 Volumes required to generate a low calibration curve ranging from 0 to 50 ng/mL for use in the Ribogreen™ vs RiboDyEE™ Assay.

Volume of Stock (0.2 μ g/mL) (μ L)	Volume of 1X TE (μ L)
50	50
40	60
25	75
10	90
5	95
2.5	97.5
1.25	98.75

0	100

6.2.4 Analytical Validation of Curves

All analytical validation calculations were performed in Microsoft Excel. RiboDyEE™ curves were monitored for precision, accuracy, LOD, LOQ, user variability and interday and intraday variability. Additionally, RiboDyEE™ was compared to Ribogreen™ using a series of clinically relevant RNA-LNP formulations, where encapsulation efficiency and mass balance were calculated as shown below:

$$Precision (\%) = \left(\frac{St.Dev\ of\ individual}{Mean\ of\ individuals} \right) * 100\%$$

$$Accuracy (\%) = \left(1 - \frac{expected - measured}{expected} \right) * 100\%$$

$$LOD = 3.3 * \left(\frac{STEYX}{slope} \right)$$

$$LOQ = 10 * \left(\frac{STEYX}{slope} \right)$$

$$Encapsulation\ Efficiency (\%) = \left(\frac{totalRNA - freeRNA}{totalRNA} \right) * 100\%$$

$$Mass\ Balance (\%) = \left(\frac{totalRNA}{theoreticalRNA} \right) * 100\%$$

6.2.5 High-Performance Liquid Chromatography – Photodiode Array Detector (HPLC-PDA)

6.2.5.1 Method Optimisation

RNA integrity and quantification were monitored using the Shimadzu HPLC system equipped with a Photodiode Array Detector with an injection volume of 10 µL and a flow rate of 0.3 mL/min. RNA was separated using a DNAPac™ Reverse Phase Column (internal diameter 3 mm, column length 100 mm) with a

detection wavelength of 254-260 nm. Oven temperature was 45 °C for Methods A and B, but 60 °C for C. Mobile phase A consisted of 0.1 M Triethylammonia (TEA) H₂O : ACN (75:25), while mobile phase B consisted of 0.1 M TEA in H₂O. Initial method optimisation looked at oven temperature as well as gradient effects as detailed in Table 6-5.

Table 6-5 Description of the gradient methods used in HPLC-PDA optimisation; ratios are representative of the mobile phase A %: mobile phase B %.

Time (minutes)	Method A (mpA:mpB)	Method B (mpA:mpB)	Method C (mpA:mpB)
-	78:22	38:62	78:22
2	73:27	78:22	73:27
9	38:62	73:27	38:62
11.5	73:27	38:62	73:27
15	73:27	38:62	73:27

6.2.5.2 mRNA Calibration Curves

Calibration curves were trialled using three mRNA species: Green Lantern mRNA (GrL), Fusion mRNA, and Firefly Luciferase mRNA (Fluc). Concentrations ranged between 2.5 µg/mL and 100 µg/mL, and samples were diluted in mobile phase B (0.1 M TEA in H₂O). The HPLC-PDA method used was method C.

6.2.5.3 HPLC-PDA Sample Preparation

mRNA-LNP samples were diluted in either mobile phase A, ACN or 2% Triton-TE at a ratio of either 1:2, 1:3, 1:5 or 1:10 (LNP sample:diluent). Additionally, 2% Triton-TE treated samples were incubated at 37 °C for 15 minutes to promote ‘bursting’ of the LNP. mRNA detection and quantification were performed using method C, as described in section 1.2.5.1.

6.2.6 Multi-Read-Out Combined Assay

A549 cells were seeded in a clear-bottom black 96-well plate at a seeding density of 0.8×10^4 cells/well using Phenol Red Free DMEM and cultured for 48 hours at

37 °C, 5% CO₂. Following this, cells were treated with Green Lantern-LNPs at concentrations of 8–0.25 µg/mL and incubated for a further 24 or 48 hours under the previous conditions. On the day of imaging, the dead control wells were killed by adding 3 µL of 10%-Triton-PBS. Hoechst 33342 Ready Flow™ Reagent was diluted by adding 2 drops to 1 mL of media, and 10 µL of stock was added to each well, and the plate was incubated for 30 minutes at 37 °C, 5% CO₂. Following incubation propidium iodide was diluted in media to achieve a concentration of 20 µg/mL, and 10 µL of stock was added to each well, and the plate was incubated at room temperature for a further 5 minutes. Prior to imaging, all media was removed from wells to minimise background fluorescence. Cell images were acquired on the EVOS M5000 microscope (Thermo Fisher Scientific, Waltham, MA, USA) at 10x magnification and the Trans bulb to monitor cell morphology. The GFP bulb to monitor expression, the RFP bulb to monitor propidium iodide signals and the DAPI bulb to monitor Hoechst 33342 signals. All gains were set at 4, and the LED and exposure were adjusted as appropriate for each plate. Fluorescence signals were quantified from images using ImageJ software v1.54.

$$\begin{aligned}
 & \textit{Cell Total Corrected Fluorescence (CTCF)} \\
 &= \textit{Integrated Density of GFP Image} \\
 &- (\textit{Area of GFP Image} \times \textit{Mean of Blank GFP Wells})
 \end{aligned}$$

6.2.7 Synthetic Cell Production

The synthetic cell manufacturing method was adapted from [247].

6.2.7.1 Lipids-in-Oil Preparation

Lipids in oil were initially prepared by mixing 25 µL of a 20 mg/mL DOPC:cholesterol (1:1) stock with 75 µL of methanol and 500 µL mineral oil. The solvent was then removed using Rotary Evaporation (IKA by VWR, Darmstadt, Germany) at 60 °C and 337 mbar vacuum for approximately 30 minutes. Following further optimisation, lipids in oil were prepared by mixing 500 µL of 20

mg/mL DSPC with cholesterol to obtain 1 mL of a 10 mg/mL DSPC:cholesterol (1:1) stock. Stocks could also be composed of DOPC:cholesterol in an identical manner. Both stocks were dissolved in ethanol, which was removed by rotary evaporation (IKA, VWR, Darmstadt, Germany) at 70 °C and 175 mbar vacuum. The lipids were then rehydrated in 1 mL of mineral oil. Where required, 5 µL of DiIC was added.

6.2.7.2 Lysate Preparation

For testing, synthetic cells were either produced using the Rabbit Reticulocyte Lysate (RRL) kit (Promega, Chilworth, UK) with the accompanying amino acid mixtures or with an in-house-produced HEK-293 cell lysate.

HEK-293 cell lysate was prepared by removing spent media from the T75 flask and washing cells with 5 mL of DPBS. Cells were then dissociated by adding 3 mL of TrypLE to the flask and incubating at 37 °C, 5% CO₂ for 3 minutes. 6 mL of MEM media was then added, and cells were centrifuged at 300 x g for 5 minutes. Cells were washed once with 1 mL of DPBS, then the pellet was resuspended in 500 µL of Pierce Lysis buffer and incubated on ice for 15 minutes. Cells were spun once more at 10,000 rpm for 10 minutes, and the supernatant was transferred to clean tubes and stored at -80 °C until use.

6.2.7.3 Solutions Preparation

The outer solution was a 2 M sucrose solution, while the inner and feeding solutions required for synthetic cell manufacture were prepared as shown in Table 6-6 and Table 6-7, respectively, for RRL tests, and in Table 6-8 and Table 6-9, respectively, for HEK lysate testing.

Table 6-6 List of Components required to make 300 µL of feeding solution for manufacturing synthetic cells using the Rabbit Reticulocyte Lysate mixtures adapted from [247].

Component	Volume Required to Make 300 µL (µL)
0.1 M KOH HEPES Buffer	25
1 M Magnesium Acetate	6.4
1 M Potassium Acetate	22.7
5.2 M Ammonium Acetate	13.6

50% w/v PEG6000	27.2
Amino Acid – Met	1.5
Amino Acid – Leu	1.5
2 M Glucose	30
Ultrapure Water	172.1

Table 6-7 List of Components required to make 300 μL of inner solution for manufacturing synthetic cells using the Rabbit Reticulocyte Lysate mixtures adapted from [247].

Component	Volume Required to Make 300 μL (μL)
0.1 M KOH HEPES Buffer	16.5
1 M Magnesium Acetate	4.2
1 M Potassium Acetate	15
5.2 M Ammonium Acetate	9
50% w/v PEG6000	18
Amino Acid – Met	1.5
Amino Acid – Leu	1.5
Rabbit Reticulocyte Lysate	102
2 M Sucrose	30
Ultrapure Water	102.3

Table 6-8 List of Components required to make 100 μL of inner solution for manufacturing synthetic cells using the HEK-293 Lysate mixture with LNPs encapsulated inside adapted from [247].

Component	Volume Required to Make 100 μL (μL)
0.1 M KOH HEPES Buffer	5.5
1 M Magnesium Acetate	1.4
1 M Potassium Acetate	5
5.2 M Ammonium Acetate	3
50% w/v PEG6000	6
Amino Acid I	5
Amino Acid II	5
ATP	1.2
GTP	2
2 M Sucrose	10
HEK-293 Lysate	34
Ultrapure Water	21.9

Table 6-9 List of Components required to make 100 μL of feeding solution for manufacturing synthetic cells using the HEK-293 Lysate mixture with LNPs encapsulated inside.

Component	Volume Required to Make 100 μL (μL)
0.1 M KOH HEPES Buffer	8.3
1 M Magnesium Acetate	2.1
1 M Potassium Acetate	7.6
5.2 M Ammonium Acetate	4.6
50% w/v PEG6000	9.1
Amino Acid I	7.6
Amino Acid II	7.6

ATP	1.8
GTP	3
2 M Glucose	10
Ultrapure Water	38.3

Amino Acid I mixture consisted of alanine, arginine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, asparagine, isoleucine, leucine, lysine, methionine, proline, serine, threonine and valine at a concentration of 1.5 M dissolved in 0.1 M KOH.

Amino Acid II mixture consisted of tryptophan, phenylalanine, and tyrosine at a concentration of 1.5 M dissolved in 0.1 M KOH.

6.2.7.4 Synthetic Cell Manufacture

In a 15 mL Falcon tube, 0.5 mL of lipids in oil was slowly added on top of 2 mL of outer solution, and the mixture was incubated at room temperature for 20 minutes. In a separate vial, 0.5 mL of inner solution was mixed with 0.5 mL of lipids in oil and vortexed for 1 minute at medium speed, then incubated on crushed ice for 10 minutes. Following incubation, the emulsion was layered onto the oil phase in the 15 mL Falcon tube.

The tube was then centrifuged at $100 \times g$ for 10 minutes, followed by an additional 10 minutes at $400 \times g$. Some tubes also underwent 5 minutes at $1000 \times g$ if no pellet was present. If a pellet was present, all supernatant was removed from the 15 mL tube, and the pellet/remaining oil was transferred to a 1.5 mL Eppendorf tube for further centrifugation at $1000 \times g$ for 10 minutes. Pellet was finally resuspended in 100 μ L of feeding solution and placed in a black, clear-bottom plate for treatment and analysis.

6.2.7.5 Synthetic Cell Treatment

Synthetic cells were treated with SM-102 LNPs encapsulating either Green Lantern mRNA or Firefly Luciferase mRNA at concentrations detailed in the

results. LNPs were either added to the inner solution or added to the well as a direct treatment.

6.3 Results

6.3.1 Determining the Ideal Standard

The Ribogreen™ assay has become the gold standard for measuring nucleic acid encapsulation in LNPs. However, the kit itself was never marketed solely to LNPs, necessitating optimisation. One key drawback is that the SOPs generally recommend using the rRNA standard that comes with the kit when building standard curves; however, many nucleic acid species result in variation in fluorescence readouts. In this section, the importance of choosing the correct standard was highlighted through standard curve construction and analytical validation. The results identify trends in both a low concentration curve which contains no Triton-TE and a high concentration curve containing Triton-TE. This is to allow quantification of the total RNA within a Ribogreen™ assay while accounting for any fluorescence quenching by the Triton X-100.

6.3.1.1 Interday Variation of a High Standard Curve

Initial analysis of the curves generated by the Glomax or POLARstarPOLARstar shows that, despite the same concentration range, the POLARstarPOLARstar fluorescence readings were higher than those from the Glomax. Figures 6-1a, b, e, and f illustrate the lines of best fit generated from the day 1 through day 6 data, and it is immediately apparent that the interday variation is large. This variation is also evident when the data is further analysed to give the LODs and LOQs (Figure 6-1c, d, g, and h). The range of LOD for the POLARstarPOLARstar and Glomax are ~56-952 ng/mL and ~48-828 ng/mL, respectively, and the range of LOQ is ~170-2885 ng/mL and ~146-2508 ng/mL, respectively. Despite variation from day to day, the values measured on a given day show similar quantification and detection capabilities, independent of the instrument used. For each line, the

coefficient of determination (R^2) was calculated, and across all days, values were >0.91 , indicating that the models can accurately determine concentration, except for day 5 poly A, which dropped to between 0.6 and 0.8. Lastly, as shown in Figure 6-1i, the repeatability values indicate that for both POLARstar and Glomax measurements, the %RSD exceeds 2%, indicating poor repeatability. However, the POLARstar curves show slightly better repeatability than the Glomax curves for both poly A and rRNA.

These results suggest that for the higher standards curves, both the POLARstar and Glomax can be used interchangeably, with slightly improved repeatability shown in the POLARstar curves – regardless of the standard used. All tested conditions yielded curves with similar LOD and LOQ, and that might meet standard linear regression requirements, such as high R^2 values. In terms of the payload used to create the standard, the rRNA standard shows better repeatability and lower interday variation.

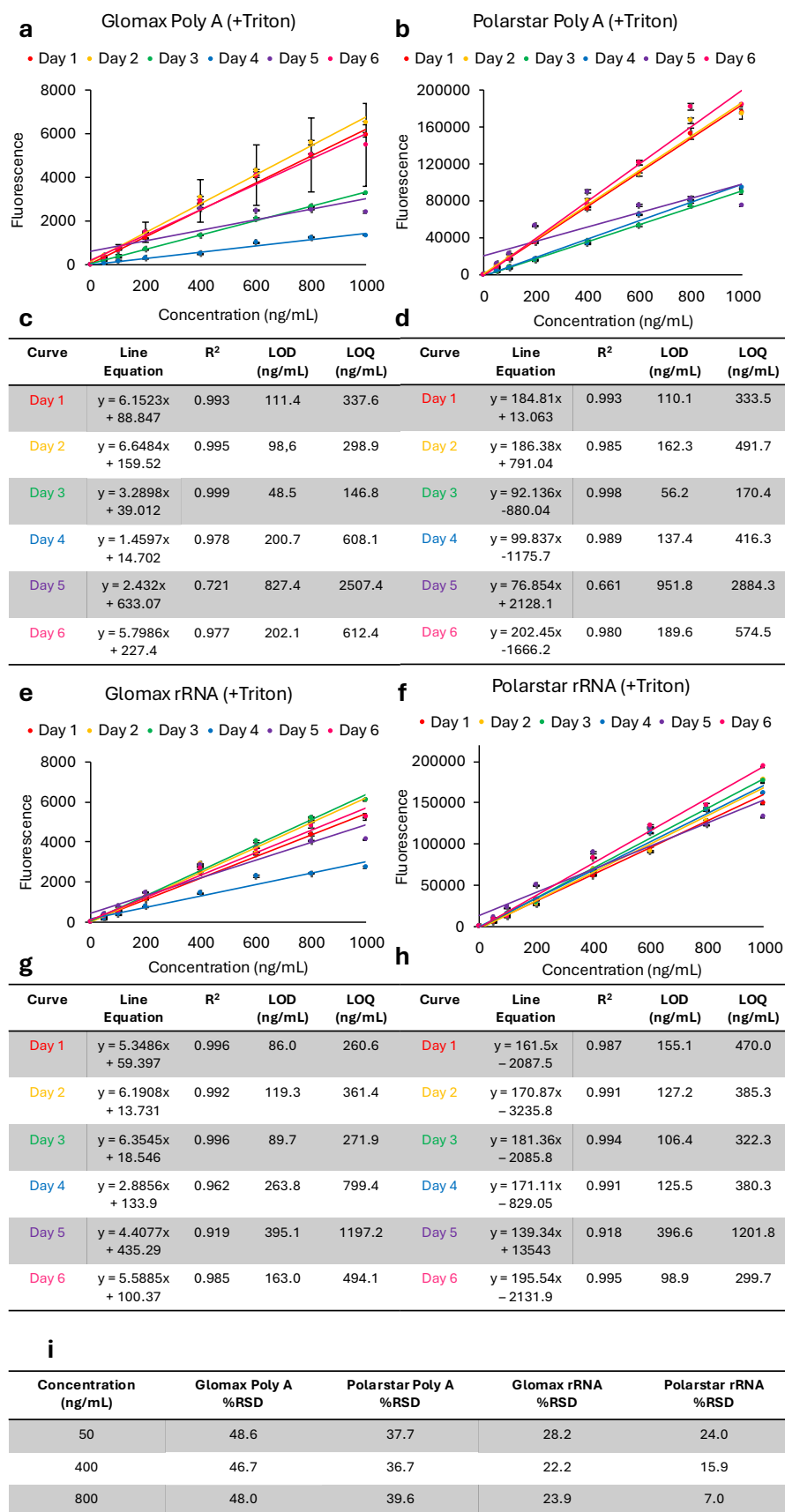


Figure 6-1 Interday Analysis of High rRNA and PolyA standard curves. Standard curves that demonstrated for poly A measured on the Glomax or Polarstar plate readers are shown in (a) and (b) while standard curves that demonstrate rRNA measured on the Glomax or Polarstar plate readers are shown in (e) and (f). For each line, figures (c-d and g-h) detail the analytical parameters that were derived from the linear regressions. With (i) finally demonstrating that repeatability at either 50, 400 or 1000 ng/mL for Day 1 curves. The standard curves are representative of the average $\pm$ standard deviation ($n=2$).

6.3.1.2 Interday Variation of a Low Standard Curve

Figure 6-2a, b, e, and f, represent the lines of best fit generated for each day, and similarly to the high standard curves, the POLARstar measurements have a higher fluorescence magnitude when compared to the Glomax measurements. Initially, results suggest again that variation is prevalent amongst the lines with the best fit; however, when linear regression analysis was performed (Figures 6-2c, d, g, and h), the LOD and LOQs of the lines are similar across most tested conditions. For poly A curves, the LODs ranged between ~54-116 ng/mL, while LOQs ranged between ~166-332 ng/mL. In contrast, for rRNA curves, the LODs ranged between ~41-112 ng/mL and LOQs between ~123-338 ng/mL. The POLARstar showed lower quantification limits in both cases, similar to the high standard curves. Days 3, 4, and 6 showed the greatest variation between the recorded LOD and LOQ limits when comparing Glomax and POLARstar. The R^2 values were lower than those of the higher curve, ranging from 0.84 to 0.98. Finally, Figure 6-2i demonstrates the %RSD for 5, 50, or 150 ng/mL across all curves. At each concentration, the rRNA standard showed lower %RSD values, indicating higher repeatability than poly A.

In conclusion, for both the high and low standard curves, caution is warranted when choosing a plate reader for analysis, and best practice is to maintain the same equipment throughout a study due to variation. While the POLARstar showed improved repeatability, the %RSD values were consistently >2%, indicating poor interday repeatability and highlighting the importance of generating a curve for each individual assay.

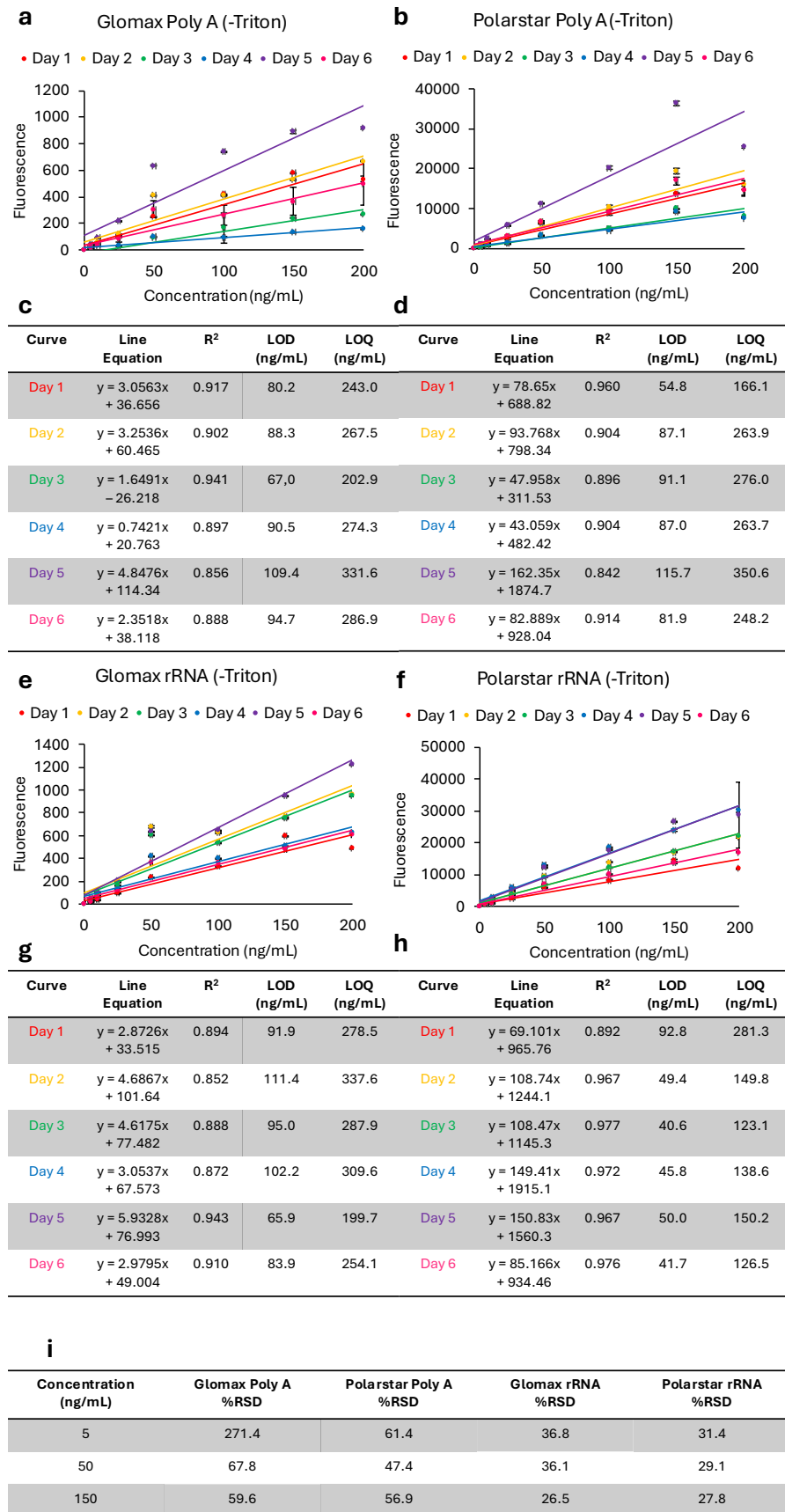


Figure 6-2 Interday Analysis of Low rRNA and Poly A standard curves. Standard curves that demonstrated for poly A measured on the Glomax or Polarstar plate readers are shown in (a) and (b) while standard curves that demonstrate rRNA measured on the Glomax or Polarstar plate readers are shown in (e) and (f). For each line, figures (c-d and g-h) detail the analytical parameters that were derived from the linear regressions. With (i) finally demonstrating that repeatability at either 5, 50 or 150 ng/mL for Day 1 curves. The standard curves are representative of the average $\pm$ standard deviation ($n=2$).

6.3.1.3 Intraday Variation of High Standard Curves

To further understand the analytical variation of nucleic acid detection curves. The high curves were generated again and measured three times in a single day to monitor intraday analytics; the results are shown in Figure 6-3.

Figures 6-3a, b, e, and f demonstrate the lines of best fit generated for each reading under each condition. Visually, repeatability is substantially increased for intraday analysis, as indicated by the overlying lines of best fit. Overall, for PolyA curves, LODs and LOQs ranged between 99-30 ng/mL and 300-430 ng/mL, respectively (Figure 6-3c and d), with minimal differences regardless of whether the Glomax or POLARstar was used. However, the rRNA standard showed greater differences between machines, resulting in wider ranges of 76-161 ng/mL and 230-487 ng/mL, respectively (Figure 6-3g and h). Regardless, all curves showed good linear fits with $R^2 > 0.98$. Additionally, increased repeatability is highlighted in Figure 6-3i, with %RSD $< 2.2\%$ except for the Glomax polyA curve at 50 ng/mL.

These results show that, for the high standard, intraday repeatability is better than interday repeatability, as indicated by lower %RSD values across all instruments and concentrations, and by similar LODs and LOQs. When examining intraday repeatability, neither poly A nor rRNA shows greater repeatability or benefits.

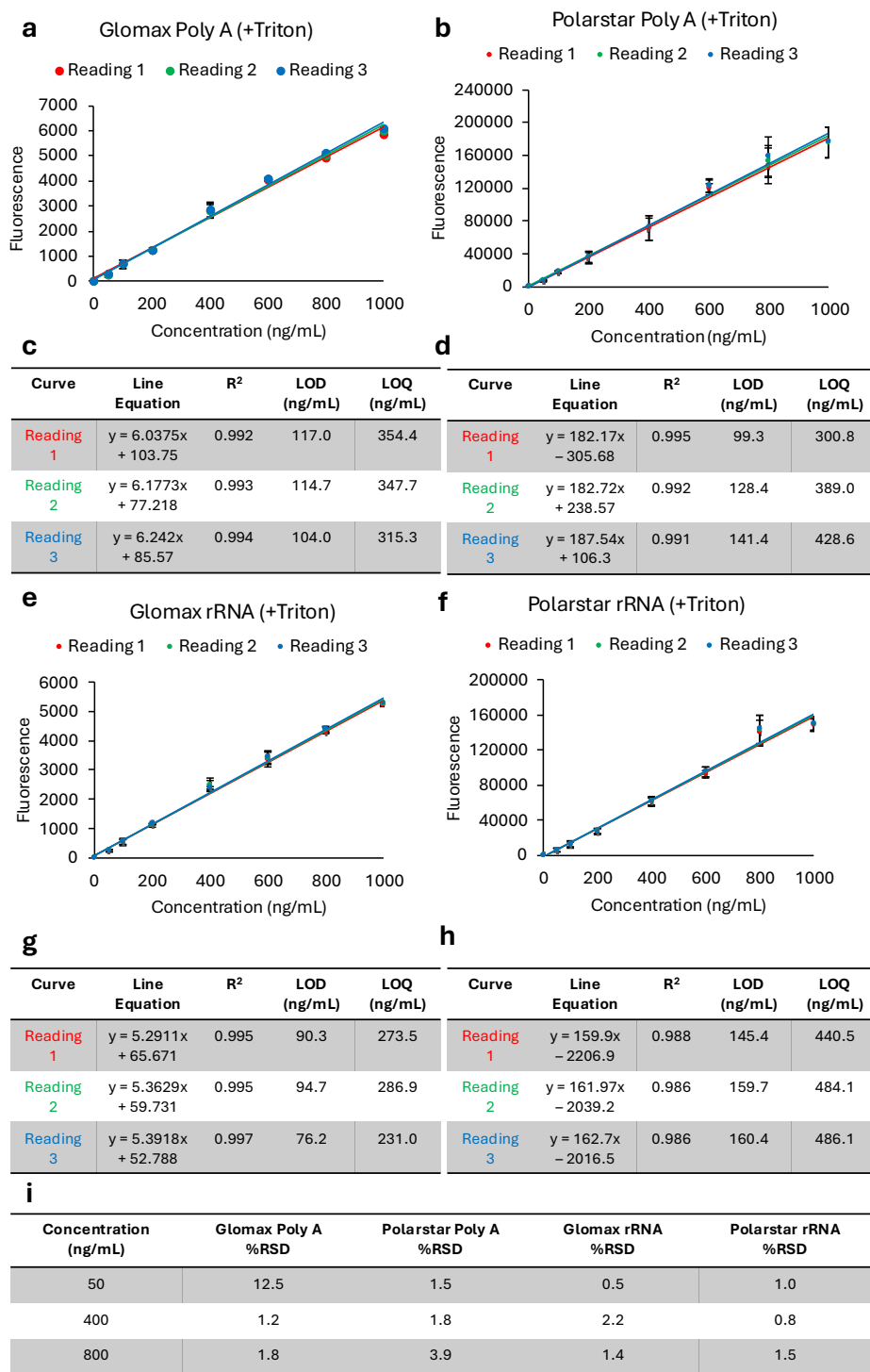


Figure 6-3 Intraday Analysis of High rRNA and Poly A standard curves. Standard curves that demonstrated for poly A measured on the Glomax or Polarstar plate readers are shown in (a) and (b) while standard curves that demonstrate rRNA measured on the Glomax or Polarstar plate readers are shown in (e) and (f). For each line, figures (c-d and g-h) detail the analytical parameters that were derived from the linear regressions. With (i) finally demonstrating that repeatability at either 50, 400 or 800 ng/mL for Reading 1 curves. The standard curves are representative of the average $\pm$ standard deviation ($n=2$).

6.3.1.4 Intraday Repeatability of Low Standard Curves

In addition to intraday analysis of high standard curves, the same analytical validation was performed on low standard curves, with results shown in Figure 6-4.

Figures 6-4a, b, e, and f demonstrate the lines of best fit generated for each reading under each condition. As in section 6.3.1.3, repeatability is substantially higher for the low curves. Overall, for poly A curves, LODs and LOQs ranged between 40-87 ng/mL and 122-261 ng/mL, respectively (Figure 6-4c and d), with the POLARstar demonstrating slightly improved quantification limits. However, the rRNA standard did not show large differences across machines, resulting in LOD and LOQ ranges of 91-94 ng/mL and 278-286 ng/mL, respectively (Figure 6-4g and h). Furthermore, poly A showed slightly improved linearity compared to rRNA, with R^2 values of 0.9-0.98 compared to 0.88-0.9. Additionally, increased repeatability was highlighted in Figure 6-4i, with %RSD <5% across all tested conditions, except at 5 ng/mL. Similar to the high standard curves, low standard curves also show enhanced repeatability when measuring analytical outputs, indicating that using the same curve for multiple plates at the same time/day could speed up assay protocols.

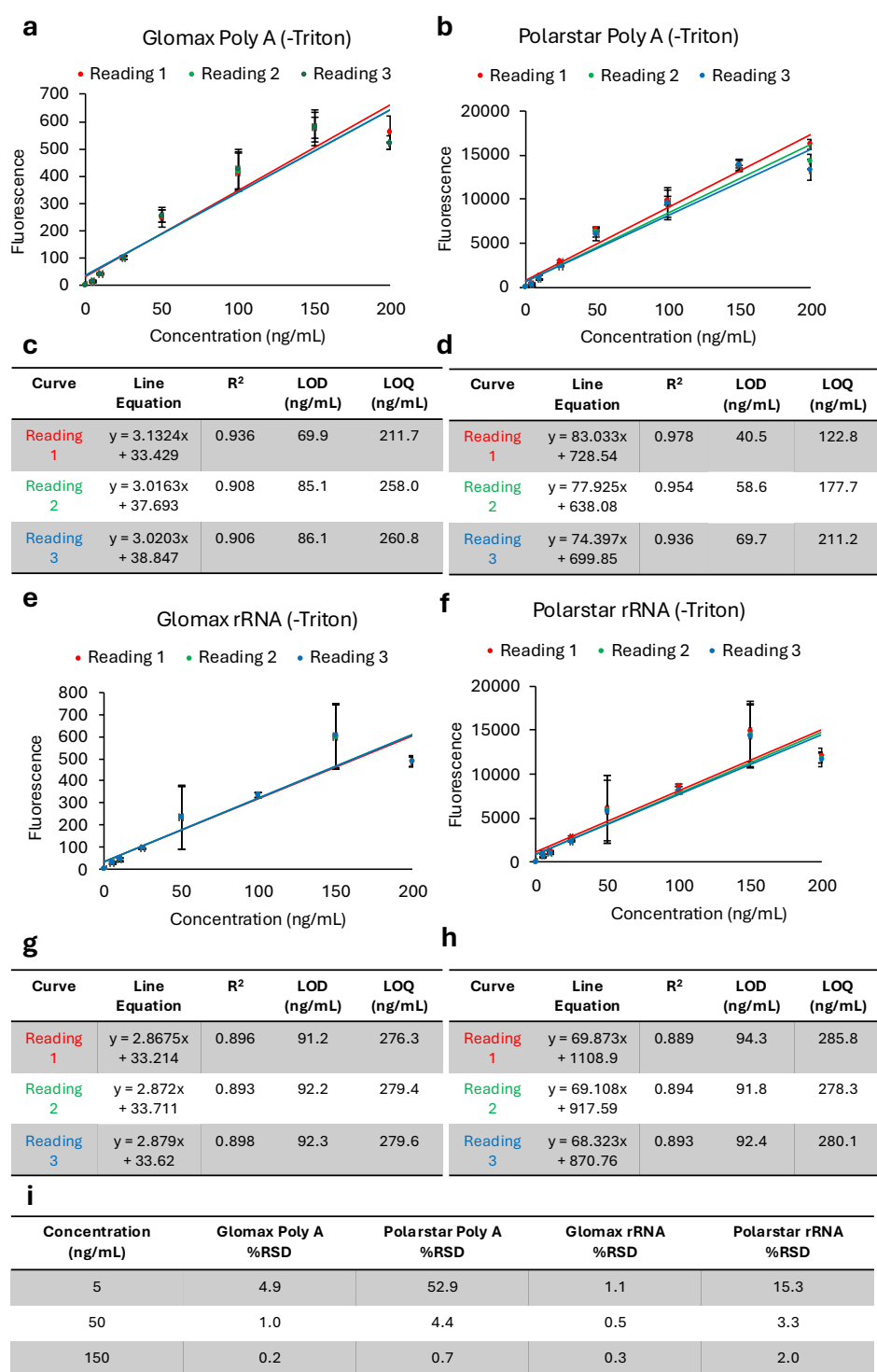


Figure 6-4 Intraday Analysis of Low rRNA and Poly A standard curves. Standard curves that demonstrated for poly A measured on the Glomax or Polarstar plate readers are shown in (a) and (b) while standard curves that demonstrate rRNA measured on the Glomax or Polarstar plate readers are shown in (e) and (f). For each line, figures (c-d and g-h) detail the analytical parameters that were derived from the linear regressions. With (i) finally demonstrating that repeatability at either 5, 50 or 150 ng/mL for Reading 1 curves. The standard curves are representative of the average $\pm$ standard deviation ($n=2$).

6.3.1.5 Accuracy of Standard Curves

To complete the analytical analysis of standard curves, accuracy was measured at three concentrations for each high and low standard curve. The results are shown in Figure 6-5.

For the high standard curves, accuracy was measured at 50, 400, and 800 ng/mL. Figure 6-5a demonstrates that, for all curves at 400 and 800 ng/mL, accuracy is good, ranging from 96-127%; however, at 50 ng/mL, accuracy is lower. With the Glomax readings for poly A and rRNA being 58.6 % and 66.0 %, respectively. This was increased in POLARstar readings at 82.0 % and 98.3%, respectively.

Similar trends are observed in the low-standard curves in Figure 6-5b, where 5 ng/mL measurements are highly inaccurate, with no accuracy. However, for the 50 and 150 ng/mL measurements, the curves were often inaccurate and overpredicted values by 111-144%. Indicating that the lower curve could benefit from optimisation of measurement values.

a

Concentration (ng/mL)	Glomax Poly A %Accuracy	Polarstar Poly A %Accuracy	Glomax rRNA %Accuracy	Polarstar rRNA %Accuracy
50	58.6	82.0	66.0	98.3
400	111.2	96.5	126.7	98.5
800	100.1	103.4	100.2	111.5

b

Concentration (ng/mL)	Glomax Poly A %Accuracy	Polarstar Poly A %Accuracy	Glomax rRNA %Accuracy	Polarstar rRNA %Accuracy
5	-142.7	-109.4	-63.1	-61.1
50	141.2	142.1	139.2	143.9
150	118.2	111.0	131.6	130.7

Figure 6-5 Accuracy of Curves at Selected Concentrations. For the high standard curve, accuracy was measured at 50, 400, and 800 ng/mL (a), while the low standard curve accuracy was monitored at 5, 50, and 150 ng/mL. Concentrations were selected to represent the low-, middle-, and high-range of the curves.

6.3.2 Validation of a New Dye: Ribogeen™ vs. RiboDyEE™

Following section 6.3.1, which demonstrated the importance of standard selection, the next step was to consider validating a new nucleic acid quantification dye, RiboDyEE™. Recognising the need for commercial suppliers to promote best practices for consistent LNP encapsulation assays, RiboDyEE™ was developed as the first commercially available dye specifically designed to measure LNP encapsulation efficiency. RiboDyEE™ can be used as a direct substitute for the industry standard, with a protocol specifically tuned for reproducible, reliable encapsulation efficiency measurements. RiboDyEE™ additionally offers a cost-effective alternative, reducing operational expenses and optimised to shorten handling time. The following results show the validation of RiboDyEE™ against the industry standard.

6.3.2.1 The Nucleic Acid Dyes Show Equivalent Analytical Performance

The study began by manufacturing three clinically relevant mRNA-LNP formulations to demonstrate that the RiboDyEE™ reagent performed equivalently in head-to-head experiments compared to the industry standard (n=3). Figure 6-6a demonstrates that similar encapsulation efficiencies (%EE) and mRNA recovery (represented as mass balance (%MB)) were achieved regardless of the reagent used. Additionally, in Figure 6-5b, c, and d, it was demonstrated that the standard curves yielded similar linear equations, with R² values >0.98. Acceptable LOD and LOQ ranges were also achieved for both reagents. Encouragingly, RiboDyEE™ reliably and reproducibly quantified encapsulated RNA in mRNA-LNP samples.

Finally, the accuracy of both reagents was assessed across six concentrations spanning each calibration curve, all of which were above their respective limits of detection. Accuracy exceeded 87% under all tested conditions (Figure 6-5e).

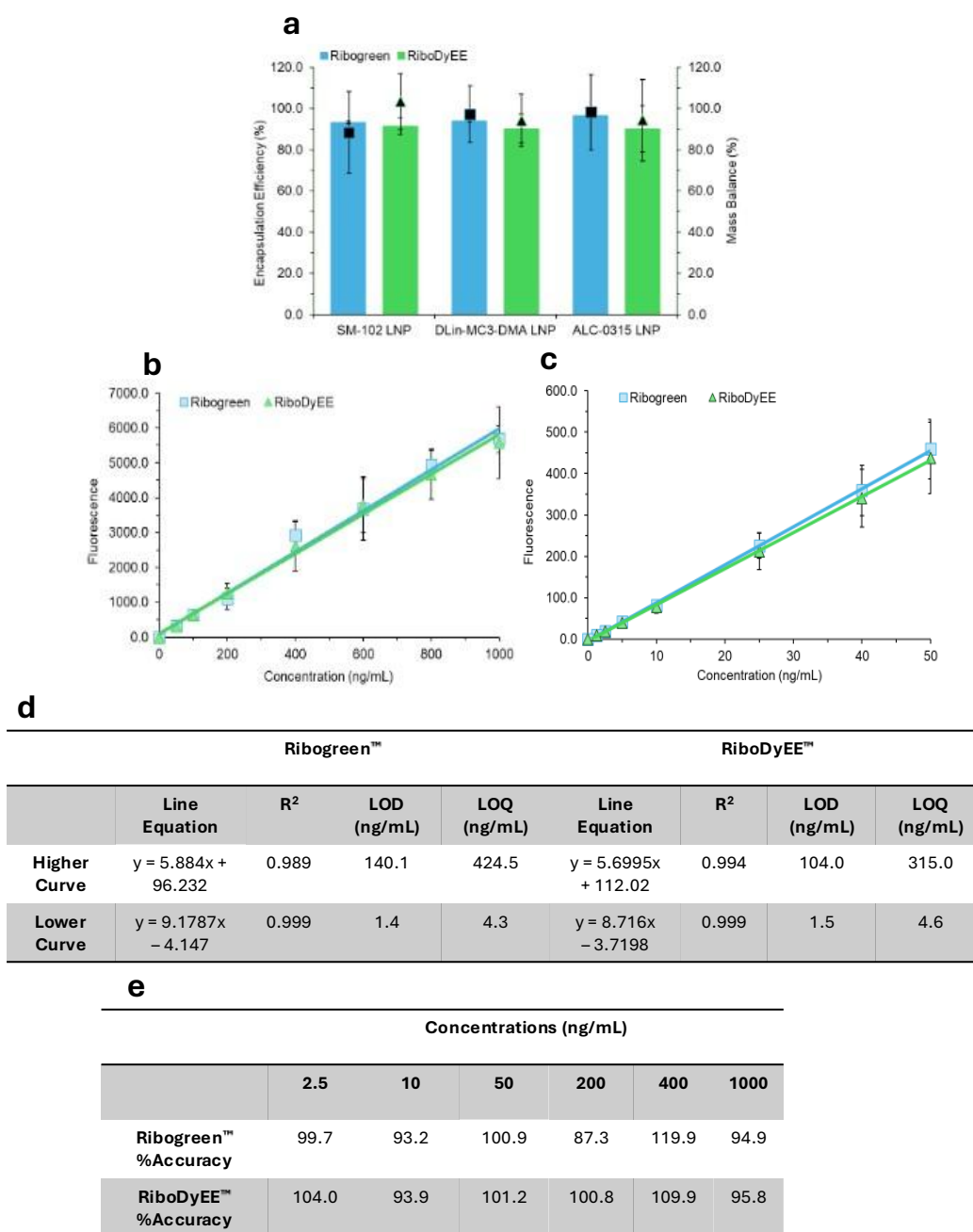


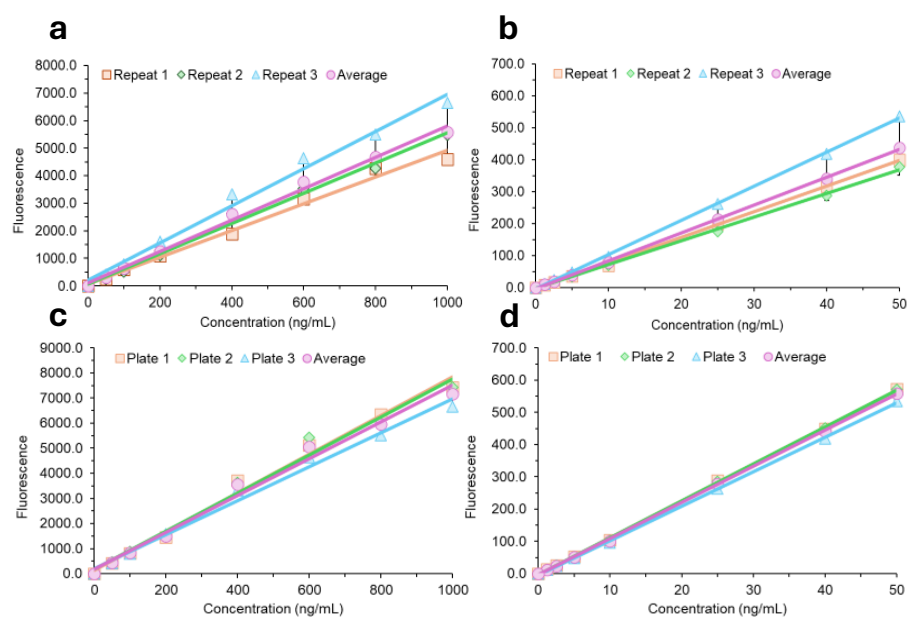
Figure 6-6 Analytical Validation of RiboDyEE™ compared to Ribogreen™. (a) shows the comparison of Ribogreen™ and RiboDyEE™ performance with encapsulation efficiency (%) represented by bars, while the mass balance is represented by triangles or squares (%). (b) represents the higher standard curves ranging between 0-1000 ng/mL while (c) represents the lower standard curve ranging 0-50 ng/mL. (d) shows the calibration parameters for each curve analyse from the linear regression while (e) shows the calculated accuracy at a range of concentrations across the high and low curves. Results are representative of the mean $\pm$ standard deviation (n=3).

6.3.2.2 Intra- and Inter-day Measurement Validation

To further demonstrate the reliability and reproducibility of RiboDyEE™, the inter- (Figure 6-7a,b) and intra-day variability (Figure 6-7c,d) of assay measurements. Interday analysis consisted of three independent curves being generated fresh on three separate days (Repeat 1-3) and measured independently, while intraday analysis consisted of three independent curves generated on the same day but on separate plates (Plate 1-3) and measured independently. Minimal intraday variation was observed; however, variability increased under interday conditions, resulting in shifts in LOD and LOQ values and changes in calibration curve equations (Figure 6-7e). User variability was also assessed (Figure 6-8), demonstrating that RiboDyEE™ produced consistent poly A standard curves across three independent users, indicating that the protocol is robust and reproducible.

Figures 6-7 and 6-8 demonstrate that standard curves generated using the RiboDyEE™ reagent are highly reproducible and reliable, not only in terms of calibration parameters such as R^2 values, but also in maintaining consistent LOD and LOQ values across testing conditions. Precision was further evaluated using the relative standard deviation (%RSD), providing insight into the clustering of individual data points around the mean. As shown in Figure 6-9, precision was generally acceptable, with %RSD values below 28%, although variability increased under interday conditions.

These findings support the recommendation to generate fresh nucleic acid standard curves for each assay when assessing LNP encapsulation efficiency to ensure reliable quantification. In addition, the influence of the RNA construct was evident, with Figure 6-7 (mRNA) exhibiting higher fluorescence intensities than Figure 6-8 (poly A), emphasising the importance of selecting an appropriate standard material. Overall, variability increased when RiboDyEE™ was applied across multiple days, reinforcing that best practice is to generate a standard curve for each individual assay to ensure accurate and reproducible results.



e

		High Curve (0-1000 ng/mL)				Low Curve (0-50 ng/mL)			
		Line Equation	R ²	LOD (ng/ml)	LOQ (ng/mL)	Line Equation	R ²	LOD (ng/ml)	LOQ (ng/mL)
Interday	Repeat 1	y = 4.8431x + 72.496	0.9883	144.4	437.6	y = 8.0408x - 3.9155	0.9995	1.6	4.8
	Repeat 2	y = 5.5055x + 56.41	0.992	119.2	361.1	y = 7.4106x - 2.8316	0.9986	2.5	7.7
	Repeat 3	y = 6.7499x + 207.14	0.9888	141.5	428.7	y = 10.696x - 4.4123	0.9996	1.3	4.1
	Average	y = 5.6995x + 112.02	0.9939	104.0	315.0	y = 8.716x - 3.7198	0.9995	1.5	4.6
Intraday	Repeat 1	y = 7.7038x + 126.68	0.9893	138.3	419.2	y = 11.448x - 3.0603	0.9969	1.3	4.1
	Repeat 2	y = 7.5791x + 178.73	0.9837	169.0	518.9	y = 11.457x - 4.2348	0.997	1.2	3.6
	Repeat 3	y = 6.7499x + 207.14	0.9888	141.5	428.7	y = 10.696x - 4.4123	0.9996	1.3	4.1
	Average	y = 7.3443x + 170.85	0.9882	145.3	440.3	y = 11.201x - 3.9025	0.9997	1.2	3.7

Figure 6-7 Inter- and Intraday Repeatability of RiboDyEE™. The interday repeatability of high (a) and low (b) standard curves is demonstrated as well as intraday repeatability of a high (c) and low (d) curve. The linear regression analysis of all lines of best fit is demonstrated in (e). Each point of the curves is the mean ± standard deviation (n=3).

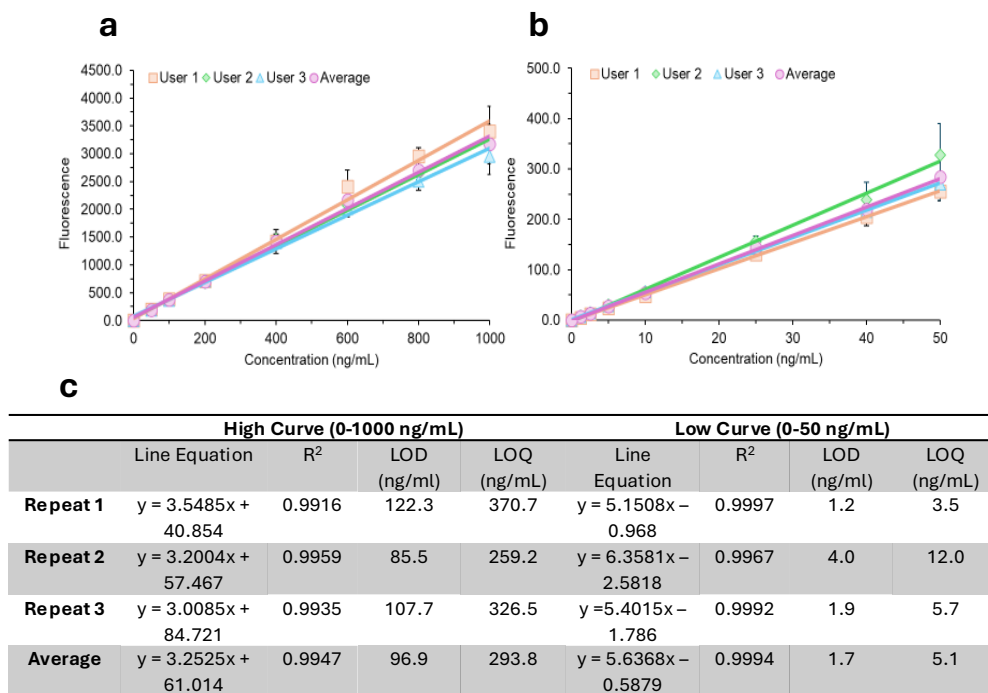


Figure 6-8 User Repeatability Testing of RiboDyEE™. Repeatability was tested over three individual users with the high (a) and low (b) standard curves represented. (c) highlights the linear regression analysis of the lines of best fit as well as the average of all three users.

		Interday	Intraday	Users
	Concentration	%RSD	%RSD	%RSD
Low Curve	2.5 ng/mL	19.4	4.1	14.0
	10 ng/mL	20.2	4.2	11.6
	50 ng/mL	19.6	3.7	16.5
High Curve	200 ng/mL	22.9	5.2	6.3
	400 ng/mL	27.5	5.7	8.6
	1000 ng/mL	18.5	6.3	11.2

Figure 6-9 Calculated Repeatability of RiboDyEE™ at specified concentrations. %RSD was measured at three concentrations on both the high and low curves to determine the accuracy of user repeats for RiboDyEE™

was developed to enable multiplexed analysis within a single plate. This approach allows simultaneous quantification of cellular expression, live/dead cell counts, and viability following LNP treatment, thereby reducing consumable use while improving the robustness and depth of the data obtained.

Initial assays were conducted on A549 cells following treatment with an LNP formulation encapsulating green lantern mRNA to enable a fluorescence readout. On the day of analysis, cell nuclei were also stained with Hoechst to

identify live cells and propidium iodide to stain dead cells. Cells were then imaged by fluorescence microscopy, and fluorescence levels were directly quantified from the images. Once completed, cells underwent a standard resazurin viability assay to obtain viability readouts. Cell images are shown in Figures 6-10 and 6-11, as well as quantified readouts shown in Figure 6-12.

Figures 6-10 and 6-11 show seamless cell staining on the EVOS M5000 microscope, allowing quantification in Figure 6-12. Cells were imaged after 24 or 48 hours of transfection. Figure 6-12a demonstrates similar GFP expression profiles across six concentrations, with higher expression at 48 hours. At 8 and 4 $\mu\text{g/mL}$ mRNA, GFP does not decrease stepwise, while Figure 6-12c indicates slightly increased PI staining, suggesting potential LNP toxicity at these doses. Figure 6-12b shows Hoechst staining at levels equal to or above those of live controls. Cell viability exceeded 100% for all tested samples (Figure 6-12d), indicating no effect from imaging or dye addition.

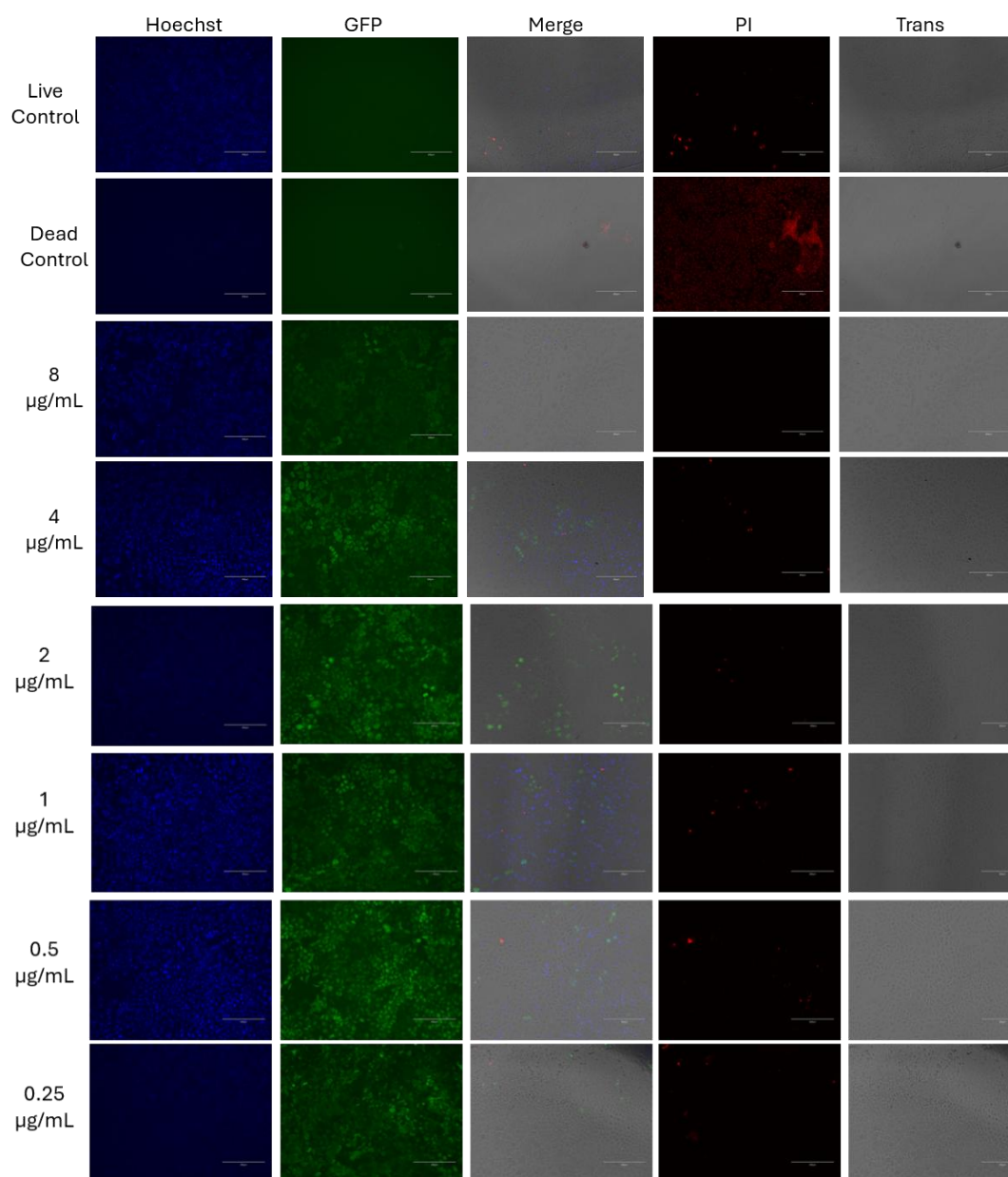


Figure 6-10 Cell Images Captured Following 24-hour Treatment. A549 cells were treated with GrL-LNPs at mRNA concentrations of 0.25 – 8 $\mu\text{g/mL}$ before being stained with Hoechst to monitor live cells and propidium iodide to monitor dead cells. Images were captured on the EVOS M5000 microscope using the DAPI, GFP, RFP, and Trans bulbs at a magnification of 10 $\times$, with gain fixed at 4. Scale bar = 300 μm .

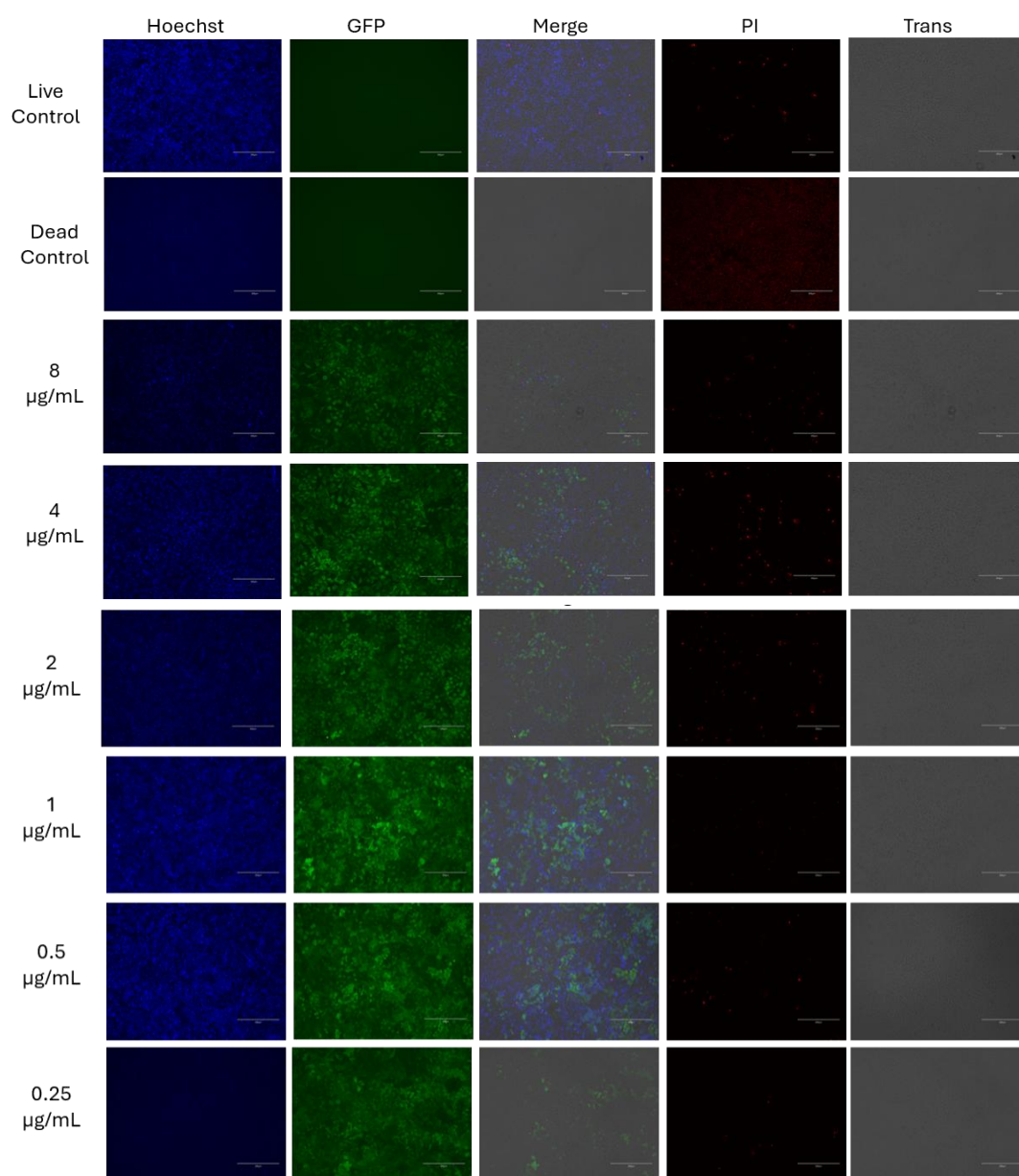


Figure 6-11 Cell Images Captured Following 48-hour Treatment. A549 cells were treated with GrL-LNPs at mRNA concentrations of 0.25 – 8 $\mu\text{g/mL}$ before being stained with Hoechst to monitor live cells and propidium iodide to monitor dead cells. Images were captured on the EVOS M5000 microscope using the DAPI, GFP, RFP, and Trans bulbs at a magnification of 10 $\times$, with gain fixed at 4. Scale bar = 300 μm .

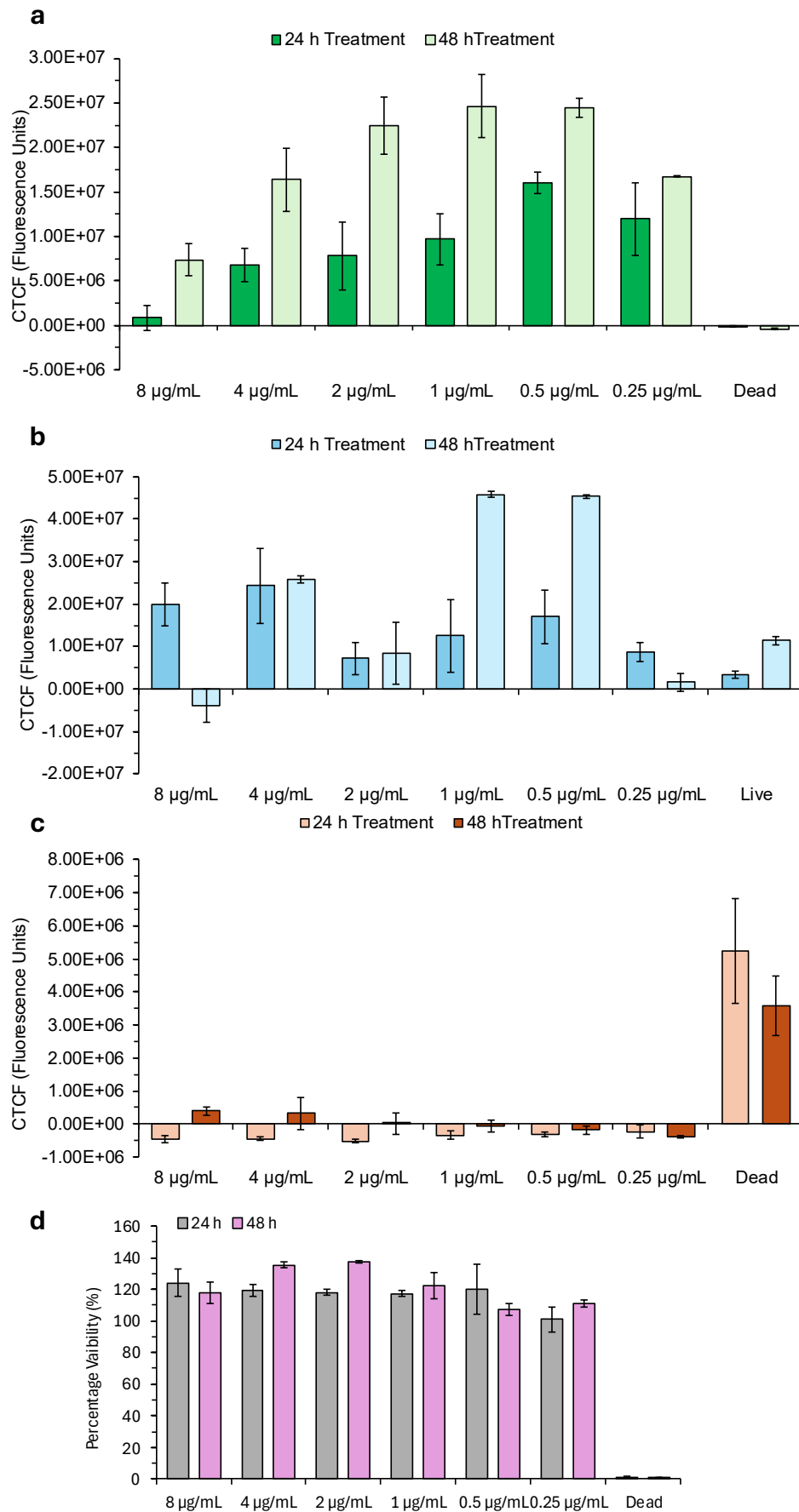


Figure 6-12 Multiple Outputs from In Vitro Assays. Fluorescence signals were quantified using Image J and are representative of the GFP expression (a), live hoechst staining (b) and dead propidium iodide staining (c) of A549 cells following 24- or 48-hour treatment with GrL-LNPs at varied mRNA concentrations. The cell viability was monitored using resazurin following cell imaging. Results are representative of the mean $\pm$ standard deviation (SD) of two independent runs.

6.3.3 HPLC-PDA Detection of mRNA in LNPs

Advancing from fluorescent dye-based assays to monitor LNP nucleic acid encapsulation would be beneficial, as it could offer a more hands-off, high-throughput, and quick method for researchers. In this section, the aim was to develop an HPLC-PDA method to detect mRNA in LNP samples, enabling quantification of concentration. Use of an HPLC-PDA method would allow researchers not only to quantify mRNA amounts but also to identify impurities, thanks to its separation capabilities. Furthermore, if LNPs encapsulate dual payloads, this could enable simultaneous quantification of both payloads.

6.3.3.1 Method Gradient Optimisation

The initial steps involved modifying pre-existing methods to achieve optimal gradient flow. To detect mRNA in LNPs, we employed a hydrophilic interaction method, in which both phases contained water and an ion-pairing agent (TEA), and one phase contained 25% acetonitrile. Four gradient methods were tested, starting with high solvent, then low solvent, and vice versa, at two oven temperatures, 45 °C and 60 °C. Naked mRNA was injected as test controls, and the resulting chromatographs are shown below in Figure 6-13.

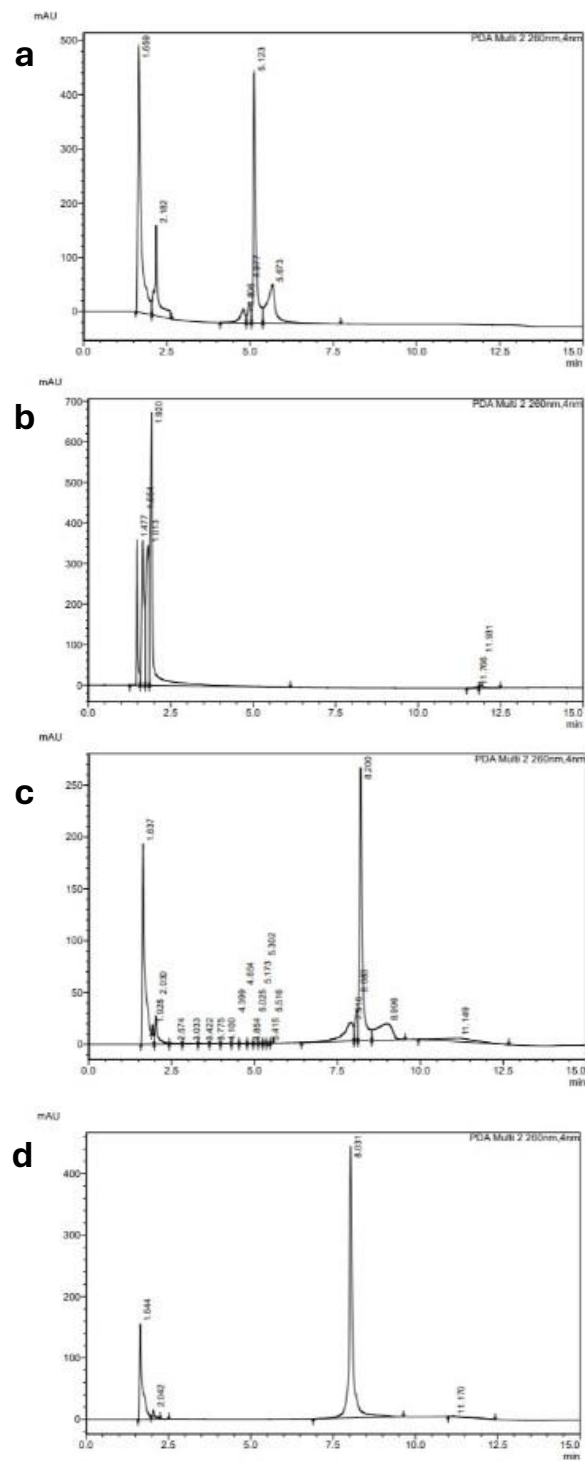


Figure 6-13 Detection of naked mRNA using HPLC-PDA. 20 μ L of a Green Lantern mRNA solution (53 μ g/mL) was injected into the column, and representative chromatographs of Green Lantern mRNA detected using method A (a), method B (b), method C (c), alongside Firefly Luciferase mRNA detected using method C (d). UV-Visible spectrum was set to 260 nm.

All methods showed an early peak within the first 2.5 minutes, which was also observed in blank runs, indicating it is unrelated to the mRNA constructs. Traditional ion-pairing chromatography begins with a low solvent concentration, allowing the mRNA to be retained on the column, and elutes it when a higher water content passes through the column. This approach was used in method B, which showed no mRNA presence (Figure 6-13b). In contrast, Figure 6-13a illustrates method A, where the phase started in a high solvent concentration and was then shifted to lower solvents; here, the mRNA construct eluted around 5 minutes (Figure 6-13a). However, in method A, it was considered that the elution peak being too close to the unidentified noise peak could impair quantification accuracy. To address this, method C was designed like method A but with an increased oven temperature of 60 °C compared to 45 °C. Theoretically, this higher temperature could help denature the mRNA strand, resulting in increased bridging with the TEA and longer retention times. It is evident that for method C, the increased temperature caused the retention time to shift to 8.2 minutes (Figure 6-13c). Furthermore, Figure 6-13d shows the same methodology applied to an alternative mRNA species, which resulted in a similar retention time of 8.0 minutes. Evidence of shoulder peaks appears for green lantern mRNA (Figure 6-13a/c), but not for Fluc mRNA (Figure 6-13d), indicating differences in purity.

6.3.3.2 Generation of Standard Curves for mRNA

The next steps in method development involved generating a standard curve to determine acceptable LOD and LOQ ranges. To do this, three mRNA constructs were examined: the previously used green lantern and Fluc mRNAs, as well as a Fluc-GrL fusion mRNA. The curves are shown in Figure 6-14.

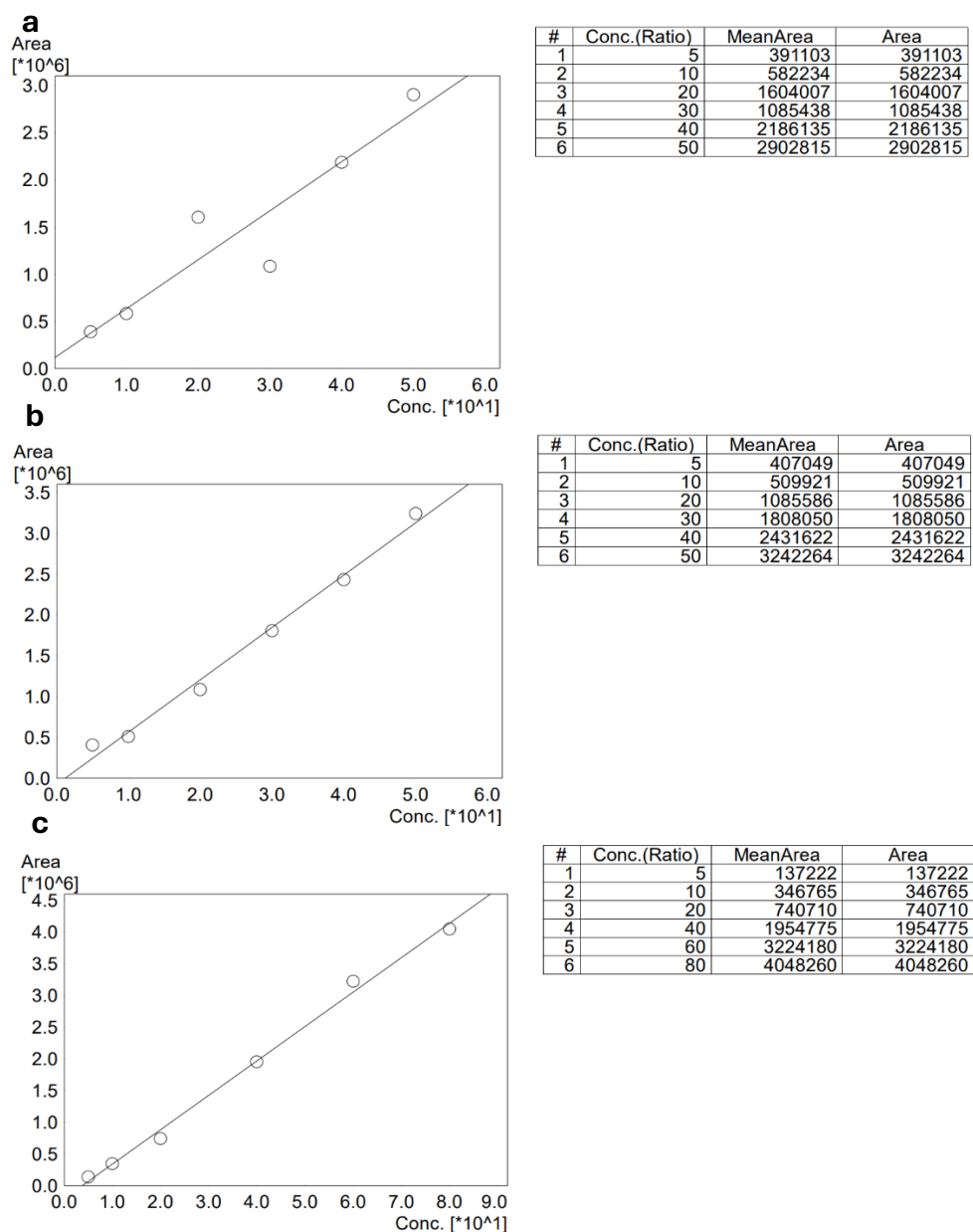


Figure 6-14 mRNA Standard Curves Generated through HPLC-PDA Quantification. Standard curves consisted of either green lantern mRNA (a), Firefly luciferase mRNA (b) or GrL-Fluc Fusions mRNA (c) at concentration ranges of 0-50 $\mu\text{g/mL}$ (a/b) or 0-80 $\mu\text{g/mL}$ (c). 20 μL of each mRNA concentration was injected into the column and run on method C, with the PDA set to a UV-Visible wavelength of 260 nm. Curves represent the area from a single measurement.

Figure 6-14 highlights that through the use of method C from section 6.3.4.1, standard curves were successfully generated for three mRNA constructs. Figure 6-14a shows the GrL curve, with some variability at the midpoints, possibly due

to the shoulder peaks observed in Figure 6-13a affecting area determination. Hence, when developing the method, it would be vital to set strict detection limits. Due to this variation, the R^2 value is 0.8745 with an LOD of 24 $\mu\text{g/mL}$ and an LOQ of 74 $\mu\text{g/mL}$. However, for both Fluc and Fusion mRNA curves shown in Figure 6-14b and c, respectively, the R^2 values are substantially improved, exceeding 0.99. Detection limits are also improved with LODs between 6-8 $\mu\text{g/mL}$ and LOQs between 19-23 $\mu\text{g/mL}$.

6.3.3.3 Determination of Sample Dilutions

Furthermore, it was crucial to identify the appropriate sample dilutions to enable mRNA release from the LNP, thereby allowing complete quantification. Initial trials examined two SM-102-based LNP samples encapsulating Fluc mRNA at concentrations of 40 $\mu\text{g/mL}$ or 80 $\mu\text{g/mL}$. The LNPs were mixed with mobile phase A at ratios of 1:2, 1:5, and 1:10 (LNP:MPA). The resulting chromatographs are shown in Figure 6-15.

Figure 6-15a shows the chromatograph for LNP samples at 40 $\mu\text{g/mL}$, while Figure 6-15b shows the chromatograph for LNPs at 80 $\mu\text{g/mL}$. In both cases, mRNA samples are eluted where expected with retention times between 7.5-8.5 minutes. Figure 6-15a shows slightly more variation in retention times, likely due to the low mRNA concentration being below the limit of accurate detection. However, all samples showed no variation in the amount of mRNA detected across the different mixing ratios. This indicated that the mixture of solvent and TEA was insufficient to fully disassemble the LNP and release the mRNA.

To address this, SM-102 and ALC-0315-based LNPs were mixed with either mobile phase A or a 2% Triton-TE mixture prior to analysis. In addition, the 2% Triton-TE samples were incubated at 37 °C to match the Ribogreen protocol. Figure 6-16a shows the chromatographs from ALC-LNPs, while Figure 6-16b represents the chromatographs from SM-LNPs. Overall, for both sample sets, mixing with 2% Triton-TE at a 1:2 ratio resulted in cleaner, larger detection peaks,

indicating that a surfactant is still required to burst the LNPs and release the mRNA for accurate quantification.

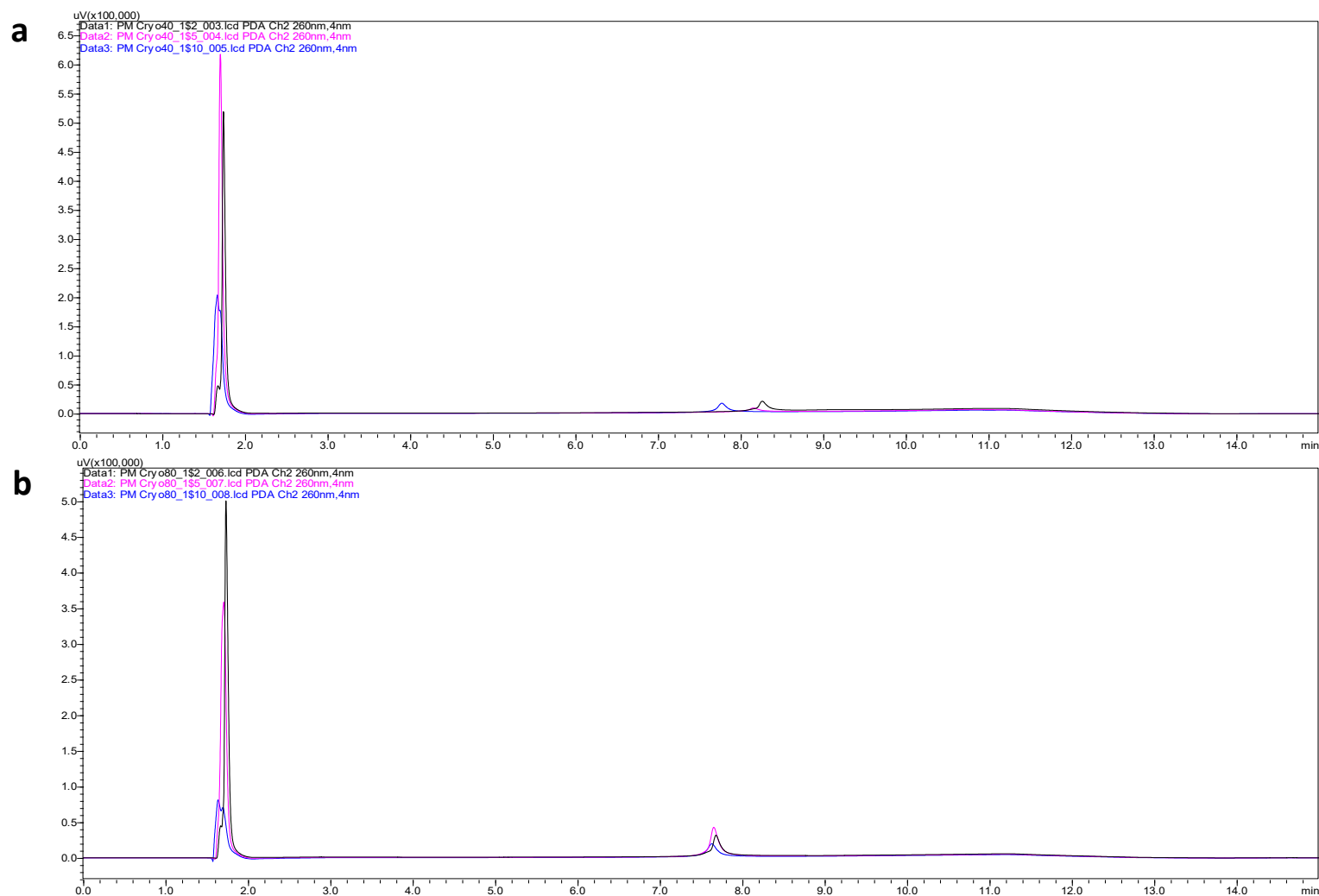


Figure 6-15 Chromatographs showing the Effect of Sample Dilution Ratios on mRNA Detection. The UV-Visible spectrum was recorded using HPLC-PDA at 260 nm. (a) highlights the spectrum achieved from LNP formulations at an initial concentration of 40 µg/mL, while (b) represents the spectrum achieved from LNP formulations at an initial concentration of 80 µg/mL. LNPs were diluted at ratios of either 1:2 (black), 1:5 (pink) or 1:10 (blue) with mobile phase A which consisted of 0.1 M TEA H₂O and Acetonitrile (75:25). Elution times for Fluc mRNA peaks ranged between 7.5-8.5 minutes.

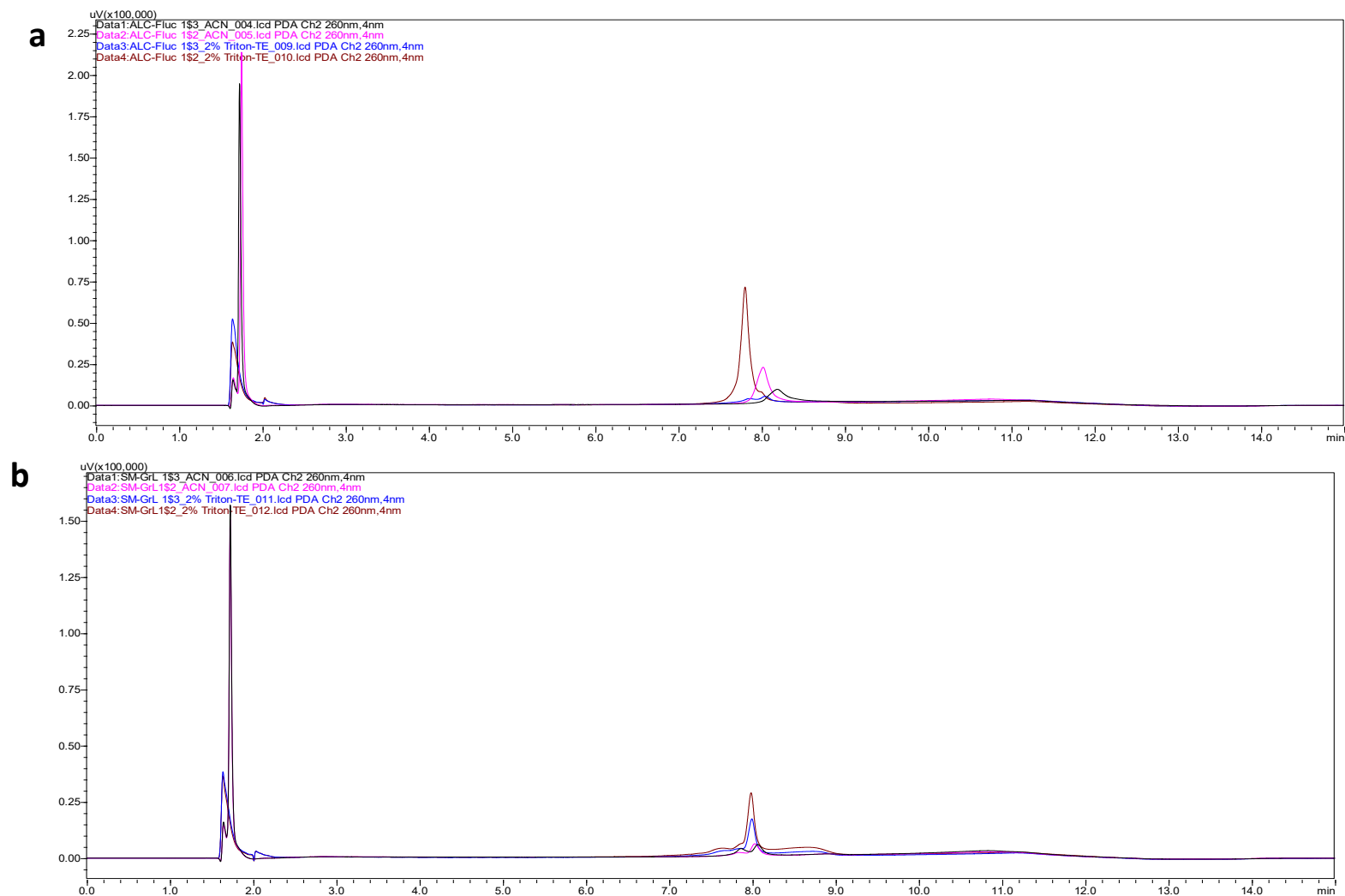


Figure 6-16 Chromatograms showing the Effect of Sample Diluent on mRNA Detection. The UV-Visible spectrum was recorded using HPLC-PDA at 260 nm. (a) highlights the spectrum achieved from ALC-LNP formulations at an initial mRNA concentration of 40 µg/mL, while (b) represents the spectrum achieved from SM-102- LNP formulations at an initial mRNA concentration of 40 µg/mL. LNPs were diluted as follows: ratios of either 1:2 (pink) or 1:3 (black) in mobile phase A which consisted of 0.1 M TEA H₂O and Acetonitrile (75:25) or at ratios of 1:2 (brown) or 1:3 (blue) with 2% Triton-TE and incubated at 37 °C for 15 minutes.. Elution times for Fluc mRNA peaks ranged between 7.5-8.5 276 minutes.

6.3.3.4 Comparison to the Gold Standard Encapsulation Measurement

This section has highlighted the feasibility of using HPLC-PDA to detect encapsulated mRNA; however, the final steps are to validate the method against the industry standard RiboDyEE™ assay. To do so, 8 LNP formulations with and without varying amounts of an outer-layer targeting ligand were injected, and mRNA levels were quantified and compared with results from the RiboDyEE™ Assay. Figure 6-17 shows the chromatographs obtained from the samples, while Table 6-10 shows the comparative mRNA quantification.

Figure 6-17a highlights DMG-based LNPs, while Figure 6-17b highlights DSPE-based LNPs, and all samples showed elution of the mRNA peak at 7.8, except for DMG (black line in Figure 6-17a), which showed no detection. Furthermore, Table 6-10 shows the quantified mRNA concentrations from each method. Some samples show similar mRNA levels between detections; however, in general, the HPLC-PDA show higher and more accurate readings, as the samples should be 40 µg/mL. This indicates that further validation across a range of samples is required, and that using both techniques would be the most beneficial form of physicochemical monitoring.

Table 6-10 mRNA Quantification Comparisons between RiboDyEE™ and HPLC-PDA methods. 8 LNP formulations were tested and quantified using previously described RiboDyEE™ protocols, and HPLC-PDA was detected at a wavelength of 260 nm using method C. Results are representative of one independent repeat.

Sample Name	RiboDyEE™ (µg/mL)	HPLC-PDA (µg/mL)
DMG	33	No Peak Detected
DMG-0.3%	29	51
DMG-0.75%	29	40
DMG-1.5%	33	37
DSPE	21	41
DSPE-0.3%	33	33
DSPE-0.75%	31	37
DSPE-1.5%	25	36

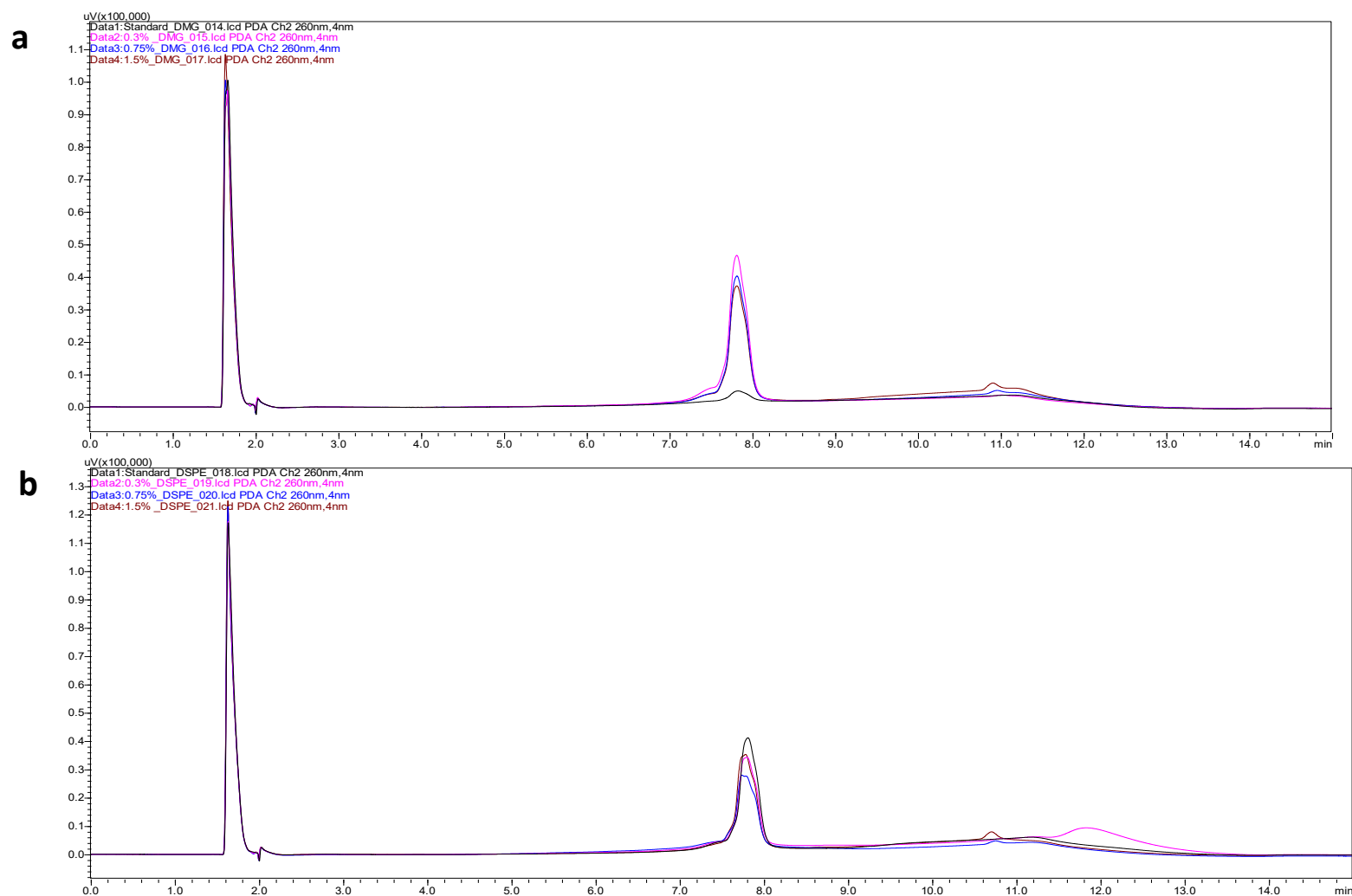


Figure 6-17 Chromatographs showing mRNA Detection from LNPs. UV-Visible spectrum was detected using HPLC-PDA at a wavelength of 260 nm. (a) highlights the spectrum achieved from DMG-LNP formulations at an initial mRNA concentration of 40 µg/mL while (b) represents the spectrum achieved from DSPE- LNP formulations at an initial mRNA concentration of 40 µg/mL. LNPs were diluted as follows: ratios of 1:2 with 2% Triton-TE and incubated at 37 °C for 15 minutes.. Elution times for Fluc mRNA peaks ranged were around 7.8 minutes.

6.3.4 Synthetic Cells as a Model for Testing LNP Efficacy

In this final results section, synthetic cells were tested as a method for monitoring LNP efficacy, reducing consumable use in *in vitro* testing, and possibly enhancing formulation selection prior to *in vivo* work.

The synthetic cells were generated by manufacturing a liposomal lipid bilayer composed of either DSPC and cholesterol or DOPC and cholesterol. The synthetic cells then had an aqueous core that contained the cellular machinery essential for protein expression, including a mixture of cell lysates, amino acids, and energy sources such as ATP. The synthetic cells were then treated with mRNA-LNPs, added externally or internally, to monitor protein expression.

6.3.4.1 Initial Treatment Trials – Naked mRNA vs. LNP

Firstly, the synthetic cells were manufactured using rabbit reticulocyte lysate from a cell-free protein expression kit. Once formulated, the synthetic cells were treated with either naked Fluc mRNA at 2 $\mu\text{g/mL}$ or an SM-102-based LNP at 2 or 20 $\mu\text{g/mL}$ to determine whether any protein expression occurred. Results are shown in Figure 6-18.

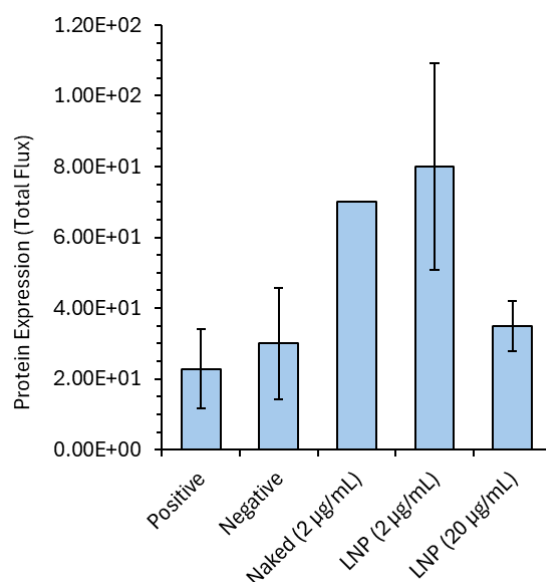


Figure 6-18 Protein Expression Monitored in CFPE and Synthetic Cell Systems. Bar graph indicates the total flux detected using ONEGlo luciferase assay substrate following treatment with either Naked Fluc mRNA or Fluc mRNA-LNPs at varying concentrations. Synthetic cells consisted of a DOPC:chol shell containing a nuclease-treated rabbit reticulocyte lysate system. Positive control indicates the lysate system treated with naked mRNA, while the negative control indicates the lysate system untreated. Treatment occurred at 37 °C for 2 hours. Results are representative of the mean $\pm$ standard deviation ($n=1-6$).

Figure 6-18 highlights that at a treatment concentration of 2 $\mu\text{g/mL}$, both naked mRNA and LNPs exhibited similar total flux levels, which are higher than those of the negative control, indicating protein expression. Additionally, this was higher than that of the 20 $\mu\text{g/mL}$. However, the positive control showed no expression, indicating that the system may not have been functioning properly.

To enhance our understanding of the choice of lysate, we decided to compare a nuclease-treated rabbit reticulocyte lysate with an untreated one. The lysates were then used to determine the effect of synthetic cell treatment alongside treatment of the lysate with either naked Fluc mRNA or Fluc-mRNA LNPs. The total flux measurements are shown in Figure 6-19.

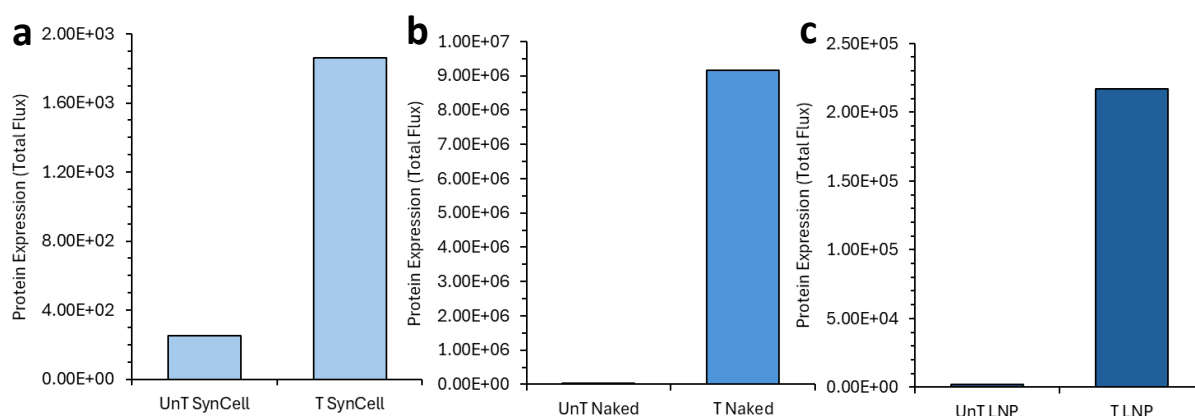


Figure 6-19 Comparison of Treatment Using a Nuclease or Non-Nuclease Treated Lysate. Testing conditions included either (a) a synthetic cell with untreated (UnT) or Treated (T) lysate and LNP treatment, (b) the lysates alone treated with naked mRNA or (c) the lysate alone treated with LNPs. Protein expression was monitored 2 hours post-incubation at 37 °C using a ONEGlo Luciferase assay substrate. Results represent the readings from one independent repeat.

Figure 6-19 demonstrates that the treated lysate shows increased expression across all treatment conditions. This could be due to the presence of host nucleic acids in the untreated lysate, resulting in low or unspecific protein expression. Additionally, Figure 6-19b shows that lysate alone treated with naked mRNA exhibits the highest protein expression, with expression decreasing in the order: lysate (naked mRNA) > lysate (LNP) > synthetic cell.

6.3.4.2 Visualisation of Protein Expression Using Synthetic Cells

Overall, results to date have shown no representative protein expression or beneficial use. Therefore, it was decided to switch to treatments with Green Lantern mRNA-LNPs to enable visual interpretation of the results. In this trial, synthetic cells consisted of a DOPC:chol core encapsulating treated rabbit reticulocyte lysate and were treated with an SM-102 based LNP encapsulating green lantern mRNA. Images were acquired pre- and post-treatment, and these are shown in Figure 6-20.

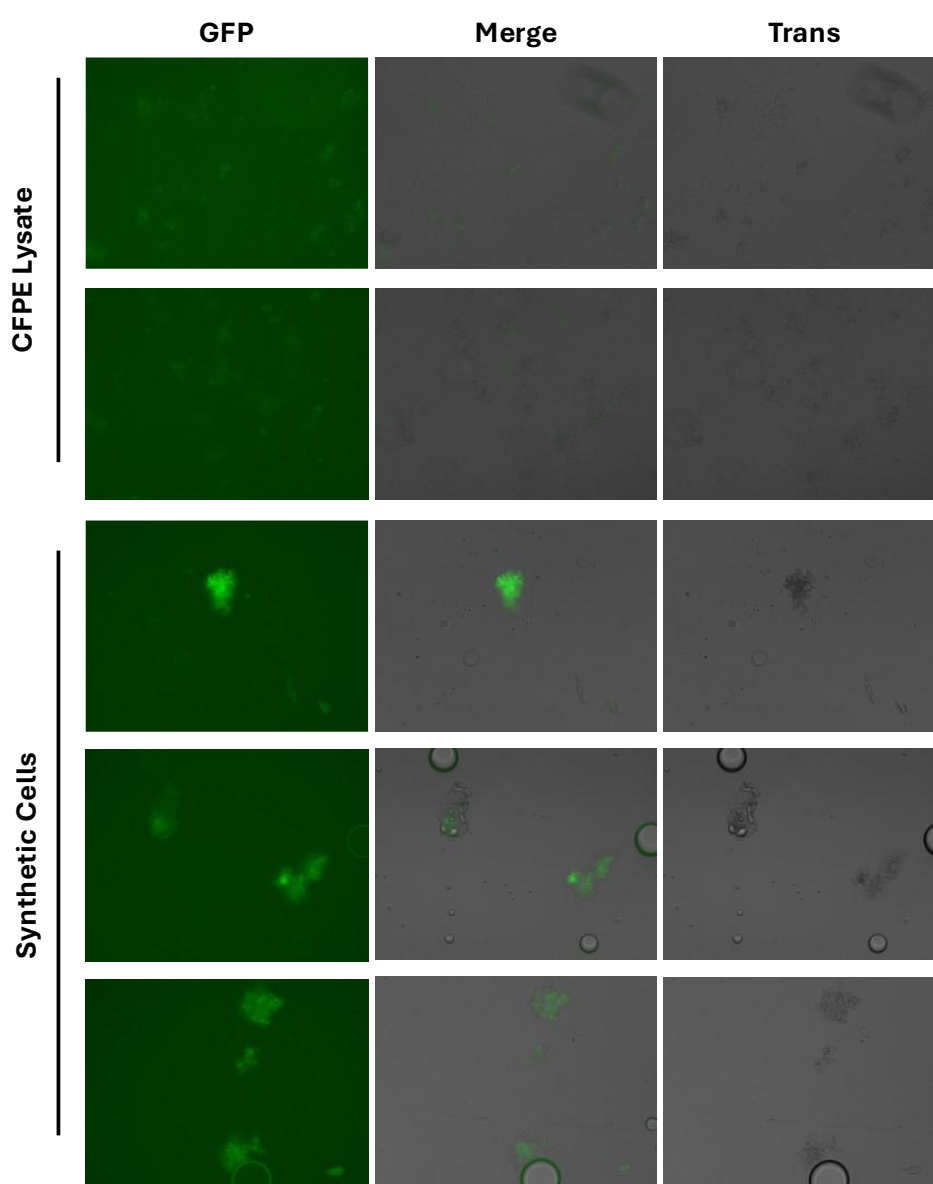


Figure 6-20 Visualisation of GFP Expression in CFPE Lysate and Synthetic Cell Systems. CFPE lysate represents the lysate alone, while synthetic cells represent the lysate encapsulating the lysate. Images are representative of the systems post-treatment with 10 μ L of Green Lantern mRNA-LNP (40 μ g/mL), incubated for 2 hours at 37 $^{\circ}$ C. Images were acquired using the EVOS M5000 microscope equipped with GFP and Trans bulbs at a magnification of 10x scale bar = 300 μ m.

Figure 6-20 shows that, for the CFPE lysate alone, no visual indicators of GFP expression were observed by imaging; however, the synthetic cell systems exhibited small clusters of expression that were brighter than the background fluorescence, indicating that expression had occurred. In an attempt to determine whether this expression was due to the presence of a synthetic cell system, the model was again manufactured as above, but this time including DiIc to stain the lipid membrane, thereby allowing visualisation. The results are shown in Figure 6-21.

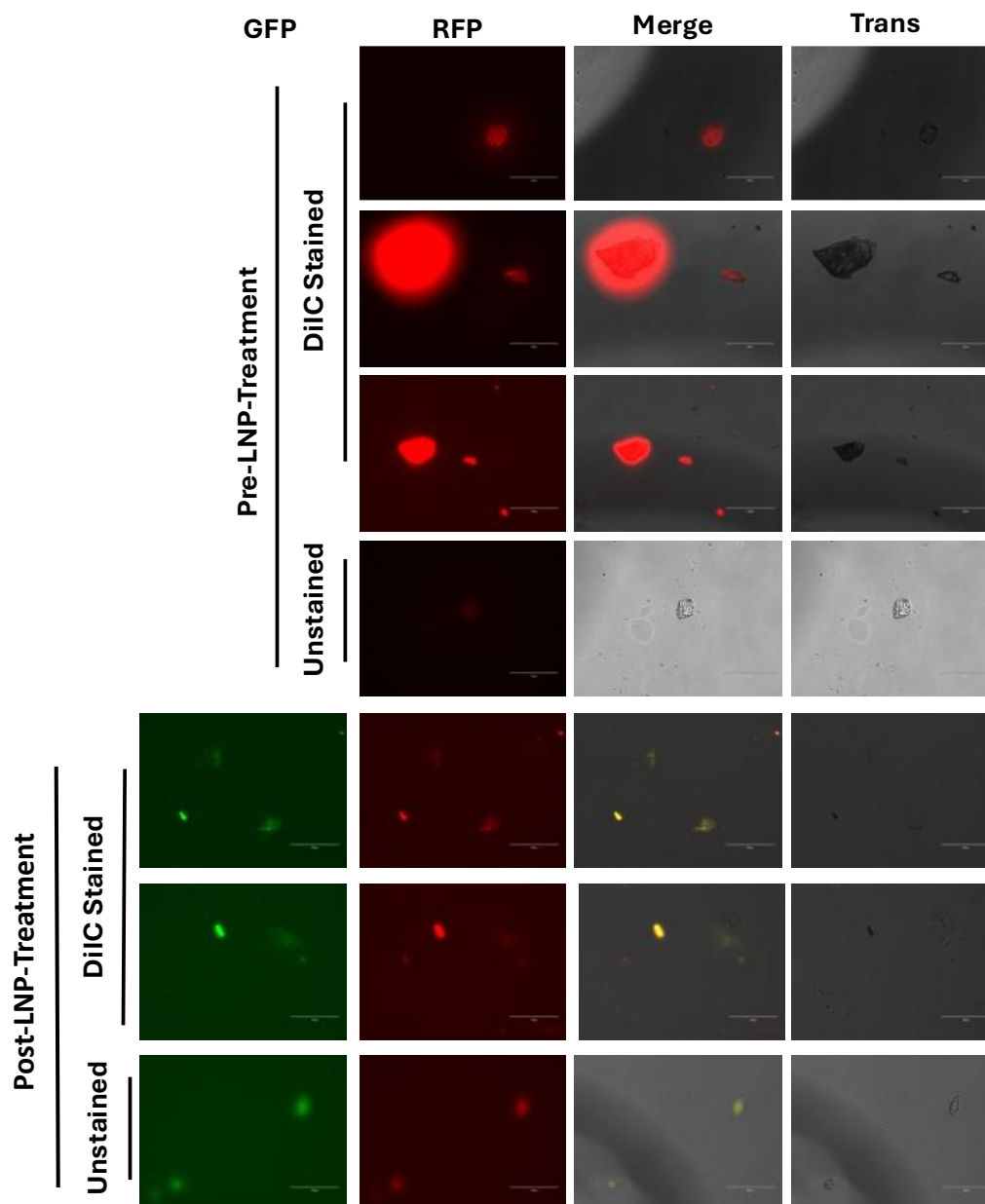


Figure 6-21 Visualisation of GFP Expression and DiLC membrane staining in Synthetic Cell Systems. Synthetic cells represent a DOPC:Chol shell encapsulating the lysate. Images are representative of the systems pre- and post-treatment with 10 μ L of a Green Lantern mRNA-LNP (40 μ g/mL), incubated for 2 hours at 37 $^{\circ}$ C. Images were acquired using the EVOS M5000 microscope equipped with GFP, RFP and Trans bulbs at a magnification of 10x/20x or 40x.

Figure 6-21 demonstrates that, prior to LNP treatment, the synthetic cells again showed evidence of large clustering, with a strong positive signal for DiIC, indicating that the clusters could contain the lipid shells and hence the synthetic cells. Furthermore, post-LNP treatment images showed evidence of GFP and RFP signals co-localising within these clusters, which again could indicate synthetic

cell production and protein expression. However, it is also vital to note that, post-treatment, the unstained control showed a slight DiIC signal, indicating that the fluorescence could also result from auto-fluorescence of the cluster.

6.3.4.3 It is What is Inside and Outside that Matters

In a final attempt to understand whether protein expression is due to synthetic cell production. We decided to manufacture synthetic cells with two different shells: either a DOPC:chol shell or a DSPC:chol shell that would encapsulate either the rabbit reticulocyte lysate used in testing so far or a HEK-293 cell lysate. Additionally, LNP treatment was carried out by either adding it post-manufacture or incorporating it into the internal solution during manufacture to assess whether protein expression increased. As previously mentioned, any changes that occurred were monitored by microscopy, and the results are shown in Figure 6-22, while Table 6-11 represents the experimental layout.

Table 6-11 Formulation Description for Synthetic Cells. Synthetic Cells were manufactured with different outer lipid shells and internal lysate mixtures, and were finally treated by adding the LNPs during or after manufacture.

Sample Number	Lipid Shell Mix	Lysate	LNP Location
1.1	DSPC:chol	Rabbit	Inside
1.2		Reticulocyte Lysate	Outside
1.3		HEK293 Lysate	Inside
1.4			Outside
2.1	DOPC:chol	Rabbit	Inside
2.2		Reticulocyte Lysate	Outside
2.3		HEK293 Lysate	Inside
2.4			Outside

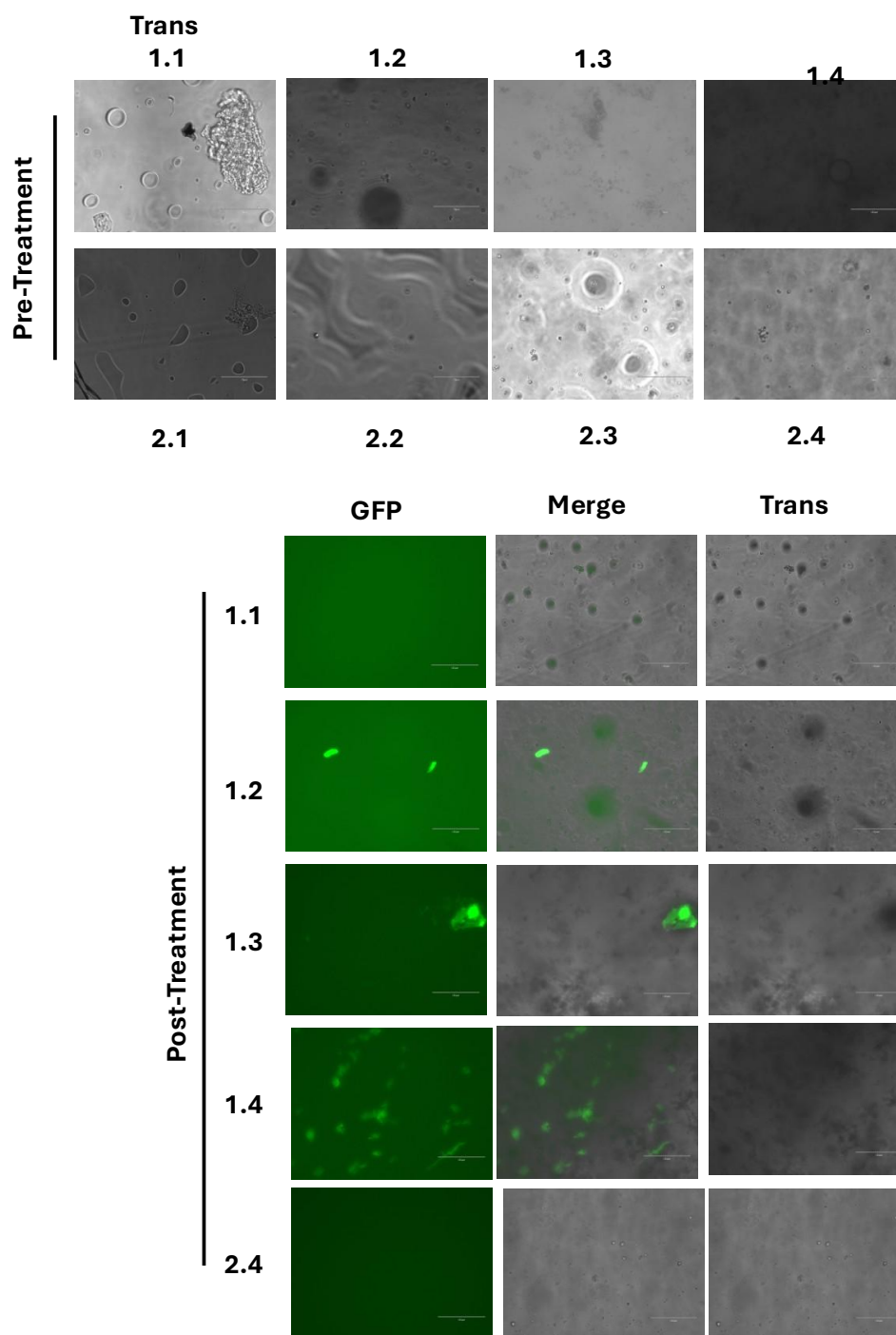


Figure 6-22 Visualisation of GFP Expression Under Different Manufacturing Conditions for Synthetic Cells. Synthetic cells represent the a DSPC:Chol or DOPC:chol shell encapsulating the rabbit reticulocyte or HEK293 lysate. Images are representative of the systems pre- and post- treatment with 10 μL of a Green Lantern mRNA-LNP (40 $\mu\text{g}/\text{mL}$) and incubated for 2 hours at 37 $^{\circ}\text{C}$. Images were acquired using the EVOS M5000 microscope equipped with GFP, RFP and Trans bulbs at a magnification of 10x, scale bar = 300 μm .

Figure 6-22 shows that, prior to treatment, synthetic cells manufactured with rabbit reticulocyte lysate (1.1/1.2/2.1/2.2) exhibited some evidence of clustering observed in previous samples. However, synthetic cells manufactured with HEK293 lysate (1.3/1.4/2.3/2.4) appear hazy, with circular shapes that seem to be in suspension, and the microscope struggles to focus on them. Post-treatment, DOPC:chol mixtures showed minimal or no GFP signal; hence, only sample 2.4 was shown as an example. However, DSPC:chol mixtures showed high GFP signal levels, with enhanced expression observed in the system using HEK293 lysate and receiving the LNP as an external treatment.

The ongoing research on synthetic cells has demonstrated protein expression in numerous samples utilising both Fluc and Green Lantern mRNA. Nevertheless, further verification is necessary to determine whether the observed expression originates from the synthetic cell system or is attributable exclusively to the ex vivo lysate system, which lacks lipid shell formation. Progress in this area could facilitate the development of efficient ex vitro screening tools, thereby supporting the selection of optimal formulations during optimisation.

6.4 Discussion

This chapter aimed to discuss current methods of RNA detection and quantification for LNPs and to assess whether enhanced analytical techniques could help ease the cost and time constraints associated with these methods. Additionally, techniques to monitor RNA efficacy after encapsulation within the LNP were evaluated to enable a more streamlined, faster approach to formulation screening.

Primarily, studies determined the effect of nucleic acid payload on binding on fluorescence output post-binding. This was monitored using polyA, and the rRNA standard included with the Quanti-iT™ Ribogreen™ assay kit. Results were monitored across two machines and demonstrated in section 6.3.1. Overall, poly A and rRNA standards exhibited similar fluorescence intensities but could yield

variations in detection limits, particularly when measured on different fluorescence plate readers. A key study on the development of Ribogreen™ was carried out by Jones et al. [15], who demonstrated that rRNA and poly A exhibited similar detection sensitivities; however, switching to other molecules, such as poly U and poly C, resulted in poor detection. Furthermore, Jones et al indicated that the size of the RNA fragment had no effect on the detection signals [248]. This agrees with the work highlighted in section 6.3.1; however, when mRNA and poly A were analysed using the RiboDyEE™ system in section 6.3.2, the fluorescence magnitudes varied at the same concentrations. This, in turn, could be caused by the mRNA strands being larger and having more binding sites present for the RNA, resulting in increased fluorescence output.

Following on in section 6.3.2, a new fluorescence-based dye – RiboDyEE™ - was analysed in comparison to Ribogreen™ where it was able to demonstrate identical levels of linear analysis, detection limits and accuracy. Fluorescence staining of RNA is a well-established field, and the choice of dye depends heavily on the species being examined. For example, double-stranded DNA often requires the use of PicoGreen™ [249]. RNA detection in LNPs has been demonstrated using several dyes. Carneiro et al. have developed protocols with similar sensitivity using both standard Ribogreen™ and SYBR Gold dyes [250]. Additionally, Wu et al. demonstrated that SYTO RNASelect has good linear relationships with RNA with minimal interference from DNA and offers the advantage of being cell permeant this in turn could benefit LNPs by removing surfactants [251]. While RiboDyEE™ still requires access to the RNA through release from the LNP, it offers a cheaper alternative compared to industry standards while maintaining high quality control and offering a protocol specific to LNP encapsulation – something the Ribogreen™ lacked.

While fluorescent dyes remain the gold-standard method, several researchers have begun exploring ways to quantify RNA encapsulated within LNPs. Blenke et al. first examined the detection of six siRNA samples and demonstrated that

Ribogreen™ resulted in greater variation than other methods tested [252]. Following this, they highlighted that Ribogreen™ shows encapsulation efficiencies similar to those of other standard methods, such as Nanodrop and UHPLC [252]. On the other hand, Espinar et al. compared standard Ribogreen™, the membrane-permeable dye SYTO 9, and Scatter-Free Absorption Spectroscopy (SFAS) to detect mRNA in nanoparticles [20], and they demonstrated that Ribogreen™ generally detected lower RNA concentrations than SFAS [253]. This was also demonstrated using the same equipment by Le Ru et al., who noted discrepancies between quantification measurements for both [254].

In this chapter, an HPLC-PDA method was developed to detect three independent mRNA constructs and, compared to RiboDyEE™, showed more accurate readings closer to the theoretical concentrations. This method offers several advantages that fluorescence-based dyes do not, including the ability to separate multiple RNA species, information on impurities, and minimal dependence on RNA structure. However, one disadvantage of this method was that the surfactant Triton was still required to disrupt the LNPs and release the RNA. As mentioned previously, Triton carry extensive risks to humans and the environment; therefore, researchers are striving to replace it [136]. Additionally, a major drawback of HPLC methods for quantifying mRNA concentrations is that they require higher concentrations of RNAs which increase cost and result in decreased sensitivity compared to fluorescence method such as Ribogreen™ which can typically measure in the nanograms. HPLC methods in general have also gained great interest in their ability to quantify RNA from LNPs using a range of techniques, including size exclusion/ion pairing [255] and anion exchange [137]. Through the implementation of advanced mass spectrometry techniques, researchers can get precise information on mRNA quantification, nucleosides and mass [125, 256]. Furthermore, researchers are aiming to streamline the simultaneous detection of multiple LNP components. For example, Su et al.

developed a method that detected three RNA species and 12 lipid species in a single sample run [257].

Preliminary studies aimed to establish a multi-readout *in vitro* assay to not only enable understanding of LNP efficacy but also provide a rationale for why the assay may or may not have worked. It was also demonstrated with a four-point readout assay that seamless quantification of live and dead cells, GFP expression, and cell viability post-LNP treatment was possible. Traditionally, multiple readout assays usually involve complex techniques such as flow cytometry, which can increase costs. This assay allows researchers to perform all intended measurements on a single plate, keeping costs low and processes time-efficient. However, major drawbacks of multi-readout assays include increased risk of causing cell death or decreased viability specifically when undertaking prolonged imaging periods that may require media to be removed.

Finally, this chapter also established synthetic cells as a method to monitor LNP translation efficacy, showing GFP expression levels higher than those of untreated controls, utilising two different lysate mixtures. The use of cell-free systems offers a highly controllable environment and would allow simplified analysis of nanoparticles and protein expression without the complexity of cellular systems. Evidently, cell-free protein expression systems alone have already been shown to demonstrate the efficacy of RNA [165], however, this RNA often has to be extracted and lacks ‘real life’ settings and is limited in translation time due to depletion of nutrients [258]. Additionally, synthetic cells have been previously used to demonstrate protein synthesis [259, 260], however, they lack key LNP requirements such as endocytosis and protein corona formation, making their functionality and biological relevance limited. To our knowledge, there is no research which shows active uptake and expression of proteins following LNP treatment in synthetic cells. While this work is still ongoing, it is theorised that any identified protein expression is most likely occurring from unencapsulated lysate material due to the lack of endosomal escape strategies.

6.5 Conclusions

In conclusion, this chapter highlighted the common pitfalls of current RNA quantification assays and fluorescent dyes, and established alternative methods for RNA detection; however, there is also a need to develop more streamlined *in vitro* and *ex vivo* testing for LNP formulation screening. In response, standard curve optimisation was carried out, as well as the development of a new fluorescence-based assay, RiboDyEE™, which offers specific techniques for LNPs. Furthermore, a new HPLC-PDA method enabled seamless detection of multiple mRNA constructs, yielding results comparable to industry standards. Finally, LNP evaluation was reimagined through the development of multiple readout assays and a new synthetic cell system, enabling more cost- and time-efficient LNP analysis platforms.

Chapter 7: Final Conclusions

Type of Publication	☐ Published article ☐ Accepted for publication ☐ Submitted for publication ☐ Part-Drafted for publication/application notes ☒ Not applicable
Citation Details (only applicable if published)	N/A
Chapter Inclusion Details	☐ This chapter is a word-for-word reproduction of the published article ☐ This chapter contains minor revisions of a drafted application note. ☐ The majority of this chapter comprises the original publication with additional data and amendments
Statement of Author Contributions (for co-authored works only)	N/A

7.1 General Overview

The aim of this thesis was to enhanced mRNA-LNP vaccine accessibility through optimisation of formulation screening and manufacturing techniques. Furthermore, the project demonstrated the use of functionalised LNP formulations to enhance expression of modified LNP mRNA delivery systems in macrophage cell models. Orthogonal techniques were also used throughout to show how characterisation of LNPs could benefit from increased quality assessment improving reliability of techniques.

7.2 Determining Critical Process Parameters in Lipid Nanoparticle Manufacture

The CQAs and hence functionality of LNPs is partly driven by the CPPs imposed during LNP manufacture. Therefore, chapter 2 aimed to evaluate select CPPs to fully understand their effect on the resulting LNP properties. Initially, successful manufacture of ionizable and cationic lipid based LNPs was demonstrated (Figure 2-2) with the ionizable lipid SM-102 being employed for the majority of the thesis due to its clinical relevance. Overall, several CPPs were highlighted to have minimal effect on LNPs CQAs, for example, TFR (Figure 2-10, 2-11), buffer pH and selection (Figure 2-5, 2-6) and lipid concentration (Figure 2-3, 2-4). However, other key CPPs were shown to have more prominent effects, for example, Flow Rate Ratio (FRR) was identified as a key determinant of LNP size when varied between 1:1 and 3:1 (Figure 2-14), with high FRR resulting in smaller sizes, likely due to rapid changes in polarity decreasing lipid solubility and enhancing the speed of nanoprecipitation to form LNPs. Furthermore, this study demonstrated that *in vitro* and *in vivo* functionality can be maintained across multiple TFRs (Figure 2-12) but caution should be taken when adapting formulations to allow for biodistribution tracking as inclusion of fluorescent dyes altered CQAs (Table 2-1) and decreased expression (Figure 2-7). This is possibility due to changes in LNP structures and/or size leading to lowered functionality. A pilot study on an often-

underrepresented CPP – head and tail cuts was also carried out. This study demonstrated that wastage is machine dependant (Figure 2-16) as well as being affected by the run itself and whether the machine is being ‘washed’ in between samples (Figure 2-17). Overall, the work in this chapter identified appropriate CPPs for LNP manufacture as well as generating an understanding of how CPPs can be adapted for a specified CQA.

7.3 Optimising Formulation Screening Methodology

Chapter 3 aimed to streamline LNP formulation screening through a direct comparison of different screening methodologies. An OFAT approach was first undertaken to highlight key differences in formulations manufactured with altered molar ratios, identifying formulations with favourable CQAs (Figure 3-2). However, it was shown that due to the limited approach of OFAT testing it was highly likely that lipid-lipid interactions within the formulations were being overlooked resulting in restricted insight in formulation screening. The study was advanced by conducting a DoE style formulation screen on either a manually operated microfluidic mixer or an automated microfluidic mixer. This chapter helped to highlight how an OFAT approach can aid researchers in refining boundaries but true statistical power lies in DoE which would be able to select optimal ratios. Figure 3-5 and 3-7 highlighted that the lipid-response ratios vary beyond the standard ratios with manually and automated DoE LNPs producing varied correlation effects (Figure 3-9) indicating that manufacturing is a key driver in formulation success. Overall chapter 3 highlighted the fundamental differences between methods and demonstrated that shifting to function-based optimal ratios could enhance LNP performance.

7.4 Increasing Accessibility of Lipid Nanoparticle

Manufacture through Low-Cost Mixers

While microfluidic mixing has advanced in the recent years, it is vital that low-cost alternatives are available to make research-scale LNP manufacture accessible to all. Chapter 4 aimed to establish the capability of two low-cost mixers alongside pipette mixing as manufacturing methods. Initial studies highlighted nucleic acid payload as a CPP for these mixing methods, with larger or modified mRNA resulting in an increase to particle size and homogeneity (Figure 4-2). However, further orthogonal analysis of LNPs indicated that structure and CQAs were consistent across mixers (Figures 4-4, 4-6, 4-7, 4-8). Furthermore, pipette mixed LNPs were shown to have comparable *in vitro* and *in vivo* activity highlighting a simple and cheap method for LNP manufacture (Figures, 4-3, 4-5, 4-10). Ultimately, automated pipette handlers were also identified as a formulation screening tool by demonstrating agreement of expression profiles across several formulations (Figure 4-9). These results were crucial for demonstrating a wide range of more conventional and affordable microfluidic mixers that could improve accessibility.

7.5 Accessing Targeting Ability of Lipid Nanoparticles

In an attempt to improve LNP functionalisation, this thesis looked at the manufacture of a modifiable targeting ligand that could be bound through an established click chemistry reaction. Specifically, here it was modified to contain a DBCO-trisFucose molecule whereby targeting the mannose receptor present on M2-subtype macrophages. It was demonstrated that this post-insertion modification had no substantial effects on LNP CQAs (Figure 5-5) and through employing two clinically relevant LNP models, chapter 5 demonstrated increased expression in M2-macrophages as well as target specific delivery for some formulations (Figure 5-7). Furthermore, it established that there was no significant effect on the *in vivo* expression profiles of LNPs (Figure 5-8 and 5-9).

This opens up a wide possibility to target immune cells *in vivo* as well as modification of the targeting ligand to allow delivery to virtually any cell type. Easy, quick and fast modifications to LNPs will be a key driver in the future of extrahepatic targeting.

7.6 Orthogonal Techniques to Determine RNA Encapsulation and Functionality

As LNPs become more prevalent in the clinic, so will the need for advanced quality control and analytic tools – specifically for RNA encapsulation and functionality testing. To address this, chapter 6 evaluated the areas of optimisation within the current gold standard RNA encapsulation test – the Ribogreen™ assay, as well as introducing and validating a new fluorescent dye – RiboDyEE™ - which aims to be an LNP-encapsulation specific assay agent. In addition, an HPLC-PDA method was established that could offer orthogonal quality control of RNA encapsulation within LNP characterisation introducing reliability into studies. RNA functionality was also monitored through a streamlined multi-output *in vitro* assay that could determine cell live/dead, viability and expression signals in one well. Synthetic cells were also investigated as an LNP expression assay, showing promising results however time constraints means that there is still a large area of work needing to be done. In conclusion, this chapter is vital to the current research as it helps to improve the approach to understanding RNA encapsulation and biological activity.

7.7 Future Studies and Concluding Points

To conclude, this thesis was aimed at making LNP research more accessible and this was investigated through the use of statistical models such as a DoE allowing for analysing of a larger design space with minimal experimental runs, as well as improving accessibility through low-cost manufacturing methods.

LNP research could benefit from more automated formulation screening. This thesis highlighted that automated DoEs can vary from manual DoEs but importantly they reduce user to user variation. Implementation of these systems with automated characterisation such as in line DLS measurements can drastically speed up LNP screening making it easier to get medicines to market faster. This also plays into increasing scalable and accessible mixing technologies which could maintain particle consistency as they move from lab to clinical and patient.

Extrahepatic targeting could also greatly benefit LNPs through decreasing off target adverse effects as well as improving therapeutic availability. Here it was demonstrated that LNPs could be functionalised with a target moiety that could be further investigated for more specific functionalisation and hence targeting. By employing this, researchers would be able to screen, formulate and manufacture targeted formulation faster to ensure quick bench to bedside progression.

Lastly, LNP researchers would benefit from establishing rigorous and orthogonal techniques to analyse the key CQAs presenting in LNPs to ensure that appropriate acceptance ranges are set.

Overall, this thesis emphasised how the next generation of mRNA-LNPs will be developed through rational formulation, manufacturing and targeting studies.

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