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Investigating Current Delivery Vehicles for Efficient and Targeted Delivery of Therapeutic RNA and Future Perspectives

Isha Sachan – 20191095

Styis4@nottingham.ac.uk

Project supervisor: Professor Cameron Alexander

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University of Nottingham, School of Biosciences, Sutton Bonington LE12 5RD

Abstract

The development of efficient nucleic acid delivery systems is a critical aspect of modern biomedical research aimed at harnessing the potential of gene therapy. Whilst nucleic acid-based therapies have shown success in the biomedical field, they face several key limitations that restrict their effectiveness as therapeutics. A major challenge is the efficient delivery of nucleic cargo to target tissues and cells. Due to nucleic acids being large, negatively charged molecules, they face barriers such as poor cellular uptake, degradation and endosomal escape.

Overcoming these challenges to achieve efficient delivery is vital for therapeutics success. Prominent delivery systems such as lipid nanoparticles, polymer nanoparticles, and polyplexes show promise in surpassing these limitations, alongside the therapeutic potential of messenger-RNA and antisense oligonucleotides compounded with primarily lipid nanoparticles. Further research into overcoming the limitations is displayed through hypotheses of the use of polymer micelles and the proton sponge effect.

Overall, this paper aims to provide a comprehensive overview of current successful delivery systems, recent advancements, and further novel methods to overcome the delivery limitations. Understanding of the strengths and limitations of these systems alongside the emerging approaches can be optimised by researchers to further their design and foster the translation of nucleic acid-based therapies into clinical practise, ultimately providing a route for the development of treatments for a plethora of diseases.

Contents

Introduction

Due to their high selectivity, RNA therapeutics have attracted interest over the past few decades (Dowdy, 2017). RNA based therapeutics refers to the treatment or determent of diseases using ribonucleic acid (RNA) based molecules as therapeutic agents (Kim, 2022). A number of these therapeutics have shown to be efficient in treatment (Akinc et al., 2019; Dammes and Peer, 2020; Sahin et al., 2020), with the success of the messenger RNA-based vaccine used in the COVID-19 pandemic shedding light on the therapeutic potential.

RNA is a molecule that plays a crucial role in gene expression, and RNA therapeutics utilise that function to target specific proteins or genes within cells. They hold promise for treating many human diseases, however, efficacy of delivery of these therapeutics continues to be a challenge due to poor cellular delivery, stability and restricted endosomal escape across the lipid bilayer, which hinders their use as effective therapies. The ideal delivery system must be able to transport the therapeutics in high yields whilst overcoming limitations, such as shielding degradation and enabling release at the target, all which must be achieved without causing toxicity to the host (Mitchell et al*.,* 2021). The lipid bilayer allows small, neutral and slightly hydrophobic molecules (<1000 kDa) to passively diffuse across them, whilst stopping larger charged molecules, such as RNA, from crossing them. The barrier of delivery of RNA therapeutics across this bilayer remains to be a problem, with extensive research into overcoming these limitations being conducted in recent years.

The endosome plays an important role in delivering these therapeutics, with a main challenge being the delivery of the RNA molecules to their intracellular targets (*Figure 1*). The therapeutic agent is taken up by endocytosis and confined within the endosomes, which are membrane-bound cytosolic vesicles inside cells that hold enzymes that can break down cellular materials. Endosomes are acidic and contain degradation enzymes which destroy the RNA molecules, restricting their efficacy as therapeutics (Varkouhi et al., 2011). Furthermore, endosomal maturation occurs which fuses the endosomes with intracellular organelles called lysosomes, which contains a variety of hydrolytic enzymes that contribute to the further breakdown of macromolecules (Gruenberg and van der Goot, 2006).

The success of the therapeutic agent relies on escaping the endosome to reach the cytoplasm, where interactions with target mRNA occurs, before lysosome mediated digestion of therapeutics transpires. Successful endosomal escape can improve the potential of nucleic acid-based therapeutics for numerous diseases, such as cancer, viral infections and genetic disorders (Dowdy et al., 2022).

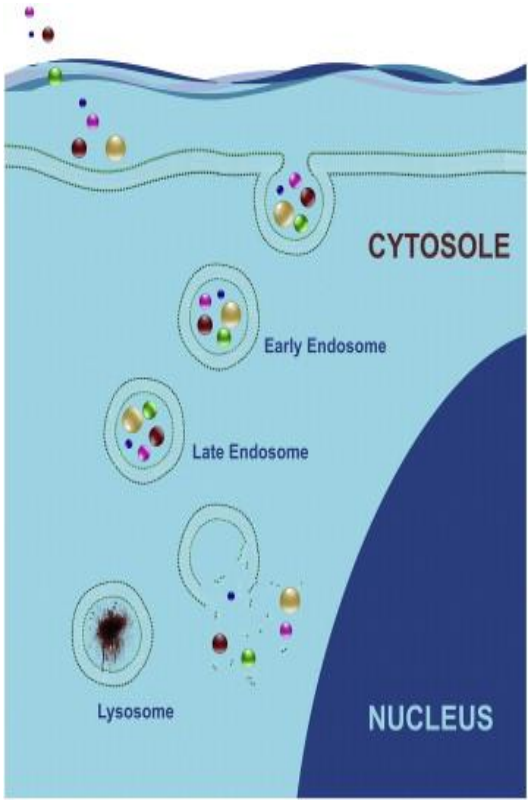


Figure 1 *- Representation of therapeutics internalising into the cell through endocytosis and subsequent endosomal escape.*

Varkouhi et al., 2011

The concept of successful small molecule drug strategy relies on the capability of nanoparticles targeting their active sites of proteins to prevent or modify their function. Research shows only ~1.5% of the human genome encodes proteins (Ezkurdia et al., 2014), and only 10-14% of those proteins have active binding sites that can be targeted by small molecules (Hopkins & Groom, 2002). The development of RNA drugs mainly focuses on two approaches; messenger RNA (mRNA), which encodes specific proteins or peptides to incur transient expression in the cytoplasm (Wolff et al., 1990); and oligonucleotide therapies, which comprise of a diverse class of drugs including antisense oligonucleotides (ASOs) which function via RNA interference (RNAi) pathways, where recognition and hybridisation of short oligonucleotides to complementary sequences occurs in RNA transcripts, altering their processing (Shen & Corey, 2017; Stephenson & Zamecnik, 1978). In particular, mRNA has shown therapeutic potential however, the limitations of endosomal escape and targeted delivery have remained a challenge when paired with some delivery systems. Lipid nanoparticles (LNPs) have been explored for the delivery of mRNA, with the most notable success being their use in the COVID-19 mRNA vaccine (Baden et al., 2021).

Research over the past few decades has ensued in secure, effective delivery systems aiding nucleic acid therapeutics, with very few being approved. The effectiveness of an RNA therapeutic depends on technologies designed and incorporated to protect against

degradation, ensure stability in blood circulation, assist in localisation to target tissue, and allow for effective intracellular delivery (Kulkarni et al., 2021).

Nanoparticles (NPs) hold capacity to improve the solubility and stability of cargos and help transportation across membranes. Recent NP designs have advanced in their ability to target agents to enhance delivery, maximise therapeutics efficiency against macromolecules, and avoid drug resistance (Wagner et al., 2018; Mitchell et al., 2020; Culver et al., 2017; Clegg et al., 2019; Cheng et al., 2020). Many therapeutics that are in late stages of development or approved are based on the technology platforms of chemically modified ASOs or LNPs.

An example of a successful RNA therapeutic is Patisiran (Onpattro) which treats hereditary transthyretin-mediated amyloidosis (hATTR). It utilises LNPs as a delivery system to deliver RNA molecules to target cells (Lim et al., 2022). The barrier of endosomal escape after cellular uptake can be avoided as Onpattro has been designed to address this limitation through a multitude of reasons, such as using LNPs with pH sensitive properties, incorporation of fusogenic lipids, and enhanced intracellular uptake mechanisms (Akinc et al., 2019).

Alternatively to LNPs, another delivery system called polyplexes are used for applications where lipid-based delivery systems may not be suitable (Tros de Ilarduya *et al*., 2010). The optimisation and design of polyplexes are crucial in accomplishing efficient and targeted delivery of RNA to the desired cells (Lächelt and Wagner, 2015). Polyplexes are complexes formed by electrostatic interactions between negatively charged RNA molecules and positively charged polymers (e.g., cationic polymers).

These delivery systems may possibly be able to surpass the limitations of endosomal escape and cellular uptake which will be further explored in this review, alongside other nucleic acid therapeutic potentials and future potential methods.

Delivery of nucleic acids

Messenger RNA

The concept of nucleic acid-based therapeutics was introduced in 1961 by Wolf *et al* through a demonstration of an injection into the skeletal muscle of a mouse of *in vitro* transcribed

(IVT) mRNA, resulting in the expression of the encoded protein in the injected muscle (Sahin et al., 2014). Since then, technologies including mRNA have been developed as an effective approach in creating new drugs that can possibly transform existing therapies or target respiratory, metabolic and autoimmune diseases, and cancer.

Amid the recent global health crisis SARS-CoV-2, the need to design suitable delivery systems for therapeutic agents was crucial. An example of a successful mRNA system is the Pfizer/BioNTech and Moderna COVID-19 vaccine, which uses mRNA to deliver the genetic sequence of the viral protein to the host cell (Machtakova et al., 2022). The expression of the virus protein is induced by the mRNA, leading to immunity. However, the mRNA cannot be used alone due to its low efficacy, as degradation by RNAses during blood circulation occurs and, due to its large size, it cannot effectively cross the cellular membrane (Houseley & Tollervey, 2009). Success of the mRNA formulation would be due to its LNP, the extracellular RNAses can be defended by the encapsulation of mRNA in cationic LNPs, which enables the uptake and consequent endosomal escape of the gene in targeted cells.

The therapeutic potential of mRNA is based on their ability to encode proteins with therapeutic activity. Due to their size, mRNAs are transcribed *in vitro* and cannot currently be made with site-specific chemical modifications using solid-state synthesis (Paunovska et al., 2022). When administered to a human, mRNA faces barriers such as to diffuse across the cell membrane due to their negative charge and hydrophilicity, evade degradation, and to have target specific delivery. To overcome these limitations. mRNAs require chemical modifications and selection of an appropriate delivery vehicle to have maximum effectiveness and efficient cellular uptake.

Molecular design improvements in mRNA's structural elements (*Figure 2*) such as the 5' cap, 3'poly(A) tail, and the 5' and 3' untranslated regions (UTRs) can affect its translation e3fficacy, stability and immunogenicity. Techniques such as elongation of the poly(A) tail, modifications to the 5' cap, engineering of the UTRs and open reading frames (ORFs) can increase the protein expression levels. Nevertheless, intracellular delivery of mRNA remains a problem

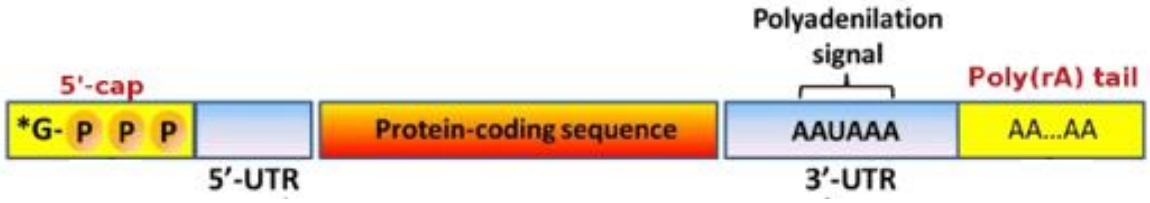


Figure 2 *– A schematic representation of mRNA (Roviello et al., 2015)*

(Kowalski et al., 2019). The current nonviral RNA delivery platform of choice are LNPs (Reichmuth et al., 2016), which can help mRNA to escape from the endosomal lumen (Paramasivam et al., 2021).

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short, synthetic nucleic acid sequences designed for binding with specific RNA targets sequentially. A typical ASO drug potential is ~20 nucleotides in length (*Figure* 3) and has a phosphonothioate linkage between nucleotides that forms the backbone (Kole et al., 2012). Additionally, five nucleotides at each flank are modified to shield the antisense oligonucleotide from exonucleases, therefore improving it stability *in vivo*. ASO were first researched to translationally repress Rous sarcoma virus (RSV) RNA to treat cytomegalovirus retinitis (Kulkarni, J.A. et al., 2021).

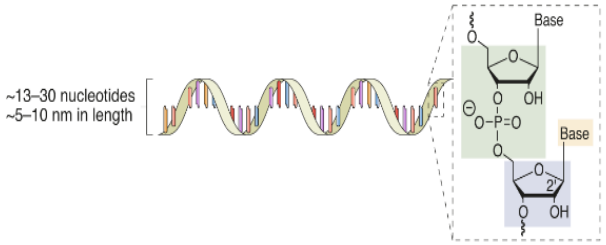


Figure 3 *– A schematic representation of an ASO, with magnification into the base structures.*

Kulkarni, J.A. et al. (2021)

ASOs serve as therapeutics as they are short RNA sequences that can degrade mRNA and inhibit protein expression by hybridizing to specific mRNA molecules. An ASO is a single-stranded sequence complementary to the mRNA sequence of the target gene. They have the capability to target any gene product of interest, and their mechanism of action is to block the start of translation or tag mRNA for degradation to avoid mRNA from being translated into protein (DeWeerdt, 2019).

Whilst they do not directly assist in endosomal escape, they can be compounded with specific delivery systems to improve their cellular uptake, followed by successful endosomal escape. ASOs can be encapsulated by LNPs which can promote endosomal escape and cytoplasmic release, allowing for interactions between the ASOs and their target RNA molecules. Possible off-target binding toxicities need to be considered for any therapeutic strategy using nucleic acids, and the molecular size and specific sequence design of ASOs make them a good therapeutic candidate compared to other nucleic acids.

They show promise for therapeutics as they can influence protein production once in the cell by binding to target mRNA, prompting degradation, which stops the translation of mRNA into a harmful protein product. The use of synthetic ASOs to regulate gene expression has been in development for the last decade, with two approved therapies being approved for the treatment of Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA) (Stein, 2016; Singh et al., 2009).

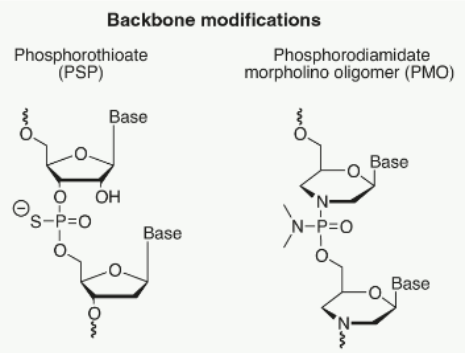


Figure 4 *– Backbone modifications of approved ASO therapeutics enhance affinity to target RNA, improve nuclease resistance, alter circulation characteristics and modulate immunological properties.*

Kulkarni, J.A. et al. (2021)

However, current limitations of ASOs *in vivo* are due to hindered cell uptake, off-target effects and a short half-life (Huang et al., 2022). *In vivo*, serum nucleases rapidly degrade unmodified phosphodiester ASOs, clearing them from blood circulation by renal filtration (Goodchild et al., 1991). Due to this, chemical alterations of the nucleobases and backbone are crucial for advancing pharmacodynamics and pharmacokinetics whilst sustaining target efficacy and affinity.

Researchers have proposed modifications that would improve the stability, such as investigations into the oligonucleotide phosphorothioate (PSP), which allows the non-bridging oxygen of the phosphate group in ASOs to be exchanged by a sulphur group (*Figure 4*), resulting in the development of a phosphorothioate bond which is resistant to nuclease-based degradation (PMO) (Liang et al., 2021).

Furthermore, there are concerns into the delivery ability of short oligonucleotides using LNPs vectors. Studies display the uptake of only 1.5-3.5% of small interference RNA (siRNA) or ASOs

into the cytoplasm occurs, with only half of the nucleotides released when endosomal disruption takes place (Gilleron et al., 2013). Thus, advances in these delivery systems to overcome issues at cellular levels are crucial for further developing oligonucleotide therapeutics (Street et al., 2022).

Effective delivery vehicles being currently researched

Lipid Nanoparticles (LNPs)

Due to the limitations outlined previously, developing an effective carrier for oligonucleotide drug delivery is necessary for the intracellular delivery of therapeutics. The current delivery system of choice are LNPs, which have been explored as vehicles for RNA particles to support intracellular delivery of oligonucleotides due to their low toxicity and relative stability (Munson et al., 2021). LNPs are spherical vesicles that contain ionisable cationic lipids alongside encapsulated nucleic acid cargo and other lipids (Hald Albertsen et al., 2022). They are positively charged at low pH levels, and neutral at physiological pH which reduce possible toxic effects (Nature Review Articles, 2021). LNPs are taken up via endocytosis, and at low pH levels, the ionizability of lipids allows for endosomal escape, granting release of cargo to the cytoplasm.

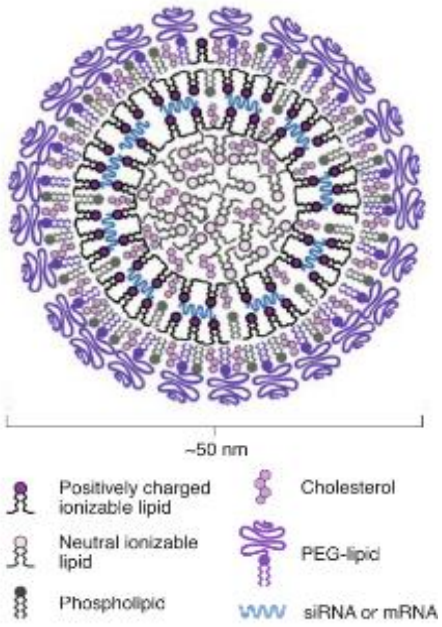


Figure 5 *– Schematic representation of an LNP containing siRNA or mRNA including key lipid components.*

Kulkarni, J.A. et al. (2021)

Studies conducted into whether the escape efficiency of LNPs depends on their distribution in several subcellular compartments have been conducted (Paramasivam et al., 2021; Gilleron et al., 2013; Wittrup et al., 2015). While another study shows conflicting results (Rink et al., 2005), results from these studies showed that escape may be restricted to early endosomal compartments before conversion into late endosomes.

They act as a defensive capsule for a nucleic acid load, preventing degradation until delivery to the target cell cytosol. They compromise of four major elements whose relative amounts affect the efficacy (*Figure 5*); PEGylated lipids for stability and circulation improvement, cationic or ionisable lipids that complex with negatively charged genetic material and assist endosomal escape, phospholipids for particle structure, and cholesterol for stability (Mitchell et al., 2020). Components of LNPs such as phospholipids (DOPE, DSPSC, etc) and cholesterol are known as 'helper lipids', which can provide structural stability and improve delivery ability by helping cytosolic entry and intracellular uptake. The type of phospholipid can influence the lipid bilayer disruption, promoting endosomal escape.

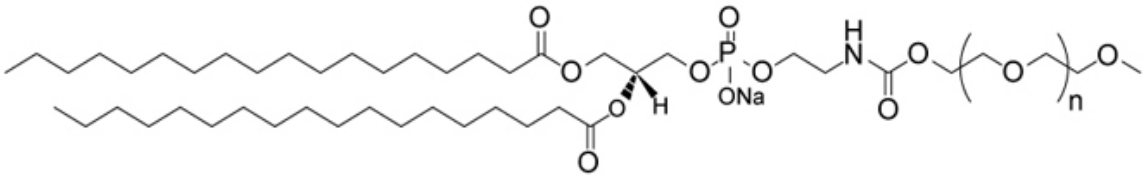
Ionisable LNPs are particularly ideal for the delivery of nucleic acid-based therapeutics as they have a neutral charge that becomes charged during acidification of the endosome, prompting endosomal escape for intracellular delivery, alongside membrane destabilisation (Patel et al., 2019). Currently, ionisable cationic lipids are considered as important parts of LNP-based RNA therapeutics due to their positive charge at low pH, which improves the encapsulation of negatively charged RNA (Jung et al., 2022), and their charge becomes more negative at physiological pH which decreases toxicity (Hou et al., 2021). The capacity to change the charge of the ionisable lipids based on the environmental pH is established as a key element in endosomal escape of LNPs. However, reports (Gilleron et al., 2013; Wittrup et al., 2015) show that less than 2-3% of nucleic acids escape the endosome to reach the cytosol, leading to improvements into this limitation being crucial to utilise the full capacity of LNPs. Nevertheless, LNPs generally show efficient encapsulation of nucleic acids, good cellular uptake, enhanced stability, and facilitates endosomal escape, making them the ideal choice of delivery system to accompany nucleic acids such as mRNA.

PEGylated lipids

A component of LNPs that, whilst constituting as the smallest molar percentage, can influence several important properties are PEGylated lipids (Hald Albertsen et al., 2022) . Polyethylene glycol (PEG) lipids are a class of PEG derivates containing lipid molecules such as DSPE or DMG (*Figure 6*). They can have effects on particle sized based on their amounts, alongside contributions to particle stability by reducing particle aggregation. Furthermore, they can

improve blood circulation times for liposome-encapsulated drugs, as well as the ability to merge particular ligands to the particle for aimed delivery. These PEGylated lipids can assist with many therapeutics, most noticeably in the LNPs used in the Pfizer-BioNTech COVID-19 mRNA vaccine.

DPSE-PEG



DMG-PEG

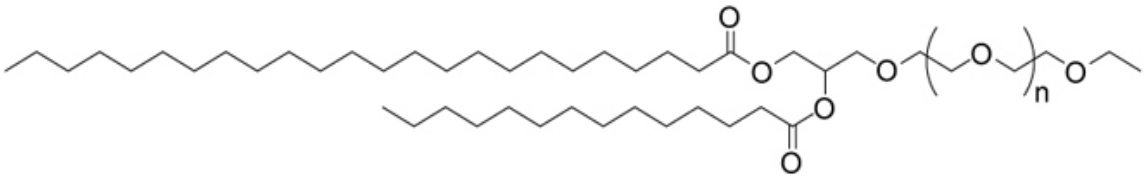


Figure 6 *- PEG Lipids used in COVID-19 vaccines, Pfizer's DSPE-PEG 2000 shown on top, and Moderna's DMG-PEG 2000 shown on bottom (BroadPharm, 2018)*

PEG-lipids influence the self-assembly of LNPs via the hydrophilic steric barrier that are constructed by PEG chains at the LNP surface (Holland et al., 1996). In LNP formation, the PEG chain expands away from the emerging particle surface, and heterogenous formations are avoided by adequate PEG-lipid gathering per particle. The steric PEG barriers also reinforce particle stability by stopping aggregation (Hald Albertsen et al., 2022). A study displayed that formulations that do not have PEG-lipids created unstable, polydisperse LNPs that surpassed 200nm in diameter (Lokugamage et al., 2021).

However, a large limitation of PEGylated liposomes is due to their high stability, they limit cellular interactions which leads to poor endosomal escape and low cellular uptake, known as the PEG dilemma (Mui et al., 2013; Harvie et al., 2000; Song et al., 2002; Hatakeyama et al., 2013). Strategies such as cleavable PEGylation (Fang et al., 2017; Juang et al., 2019), changing the percentage of PEG in formulations to 1-2% (Zalba et al., 2022), and using exchangeable PEGs have been proposed (Evers et al., 2018).

Furthermore, another limitation that requires further research is that PEG lipids are able to initially shield detection of the nanoparticles by the immune system, but as PEG extends the

life span of the particles, the immune system may be able to locate the particles and start creating an antibody response (Cross. R, 2021).

DOPE and DSPC

Another important part of LNPs that can assist in getting a molecule across the endosomal membrane, which is a critical step which requires destabilisation of the lipid bilayer structure, are two helper lipids. Dioleoylphosphatatidylethanolamine (DOPE) is a helper lipid with a relatively small headgroup, two large and unsaturated oleoyl chains, and phosphoethanolamine (PEA), resulting in its cone-like shape. This geometry can adopt the non-bilayer hexagonal (H_{II}) phase (*Figure 7*), which is found during bilayer disruption in transitional structures, which destabilises endosomal membranes and enables release of the LNPs (Hou et al., 2021).

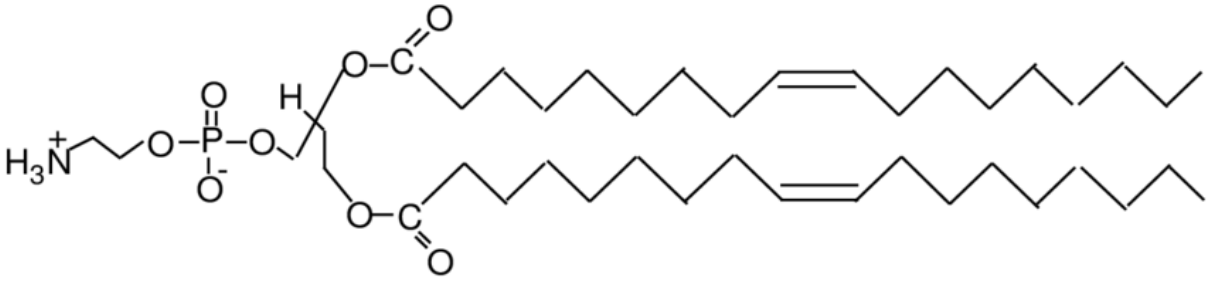


Figure 7 *– The chemical structure of DOPE (Avanti Polar Lipids)*

The phospholipid 1,2-distearyol-sn-glycero-3-phosphocholine (DSPC) contains saturated acetyl chains in the lipid tail and head group, creating a cylindrical-shaped structure (*Figure 8*). The role it plays requires further research (Kulkarni et al., 2019) however, it can enhance encapsulation efficiency and liposome stability, enabling higher effectiveness in biological systems, and due to its high transition temperature, it can prevent the premature breakdown of the LNP (Kulkarni et al., 2017).

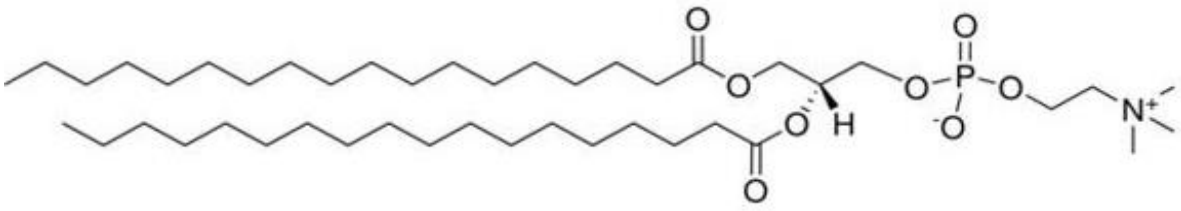


Figure 8 *– The chemical structure of DSPC (Tikhonov, Asadchikov and Volkov, 2015)*

Polymeric Nanoparticles (PNPs)

Following on from LNPs, a potential for targeted delivery of therapeutics are polymeric nanoparticles (PNPs) due to their assets provided from their small size (Rao and Geckeler, 2011). They comprise primarily of polymeric materials that can self-assemble with RNA to exhibit properties such as stability and controlled release (Nagavarma *et al*., 2012). Their advantages as drug carriers consist of their ability for target-specific delivery of therapeutics into intended positions with efficiency and their versatility, and like LNPs, modifications to their chemical structures can improve *in vivo* PNP efficiency (Madkour, 2019). PNPs are considered one of the most effective delivery systems for controlled and targeted delivery to treat primarily cancer of multiple phenotypes. However, restrictions such as low transfection efficiency, stability and degradation remain problematic, resulting in very few nanoparticle systems using PNP's, with further research required.

Furthermore, PNPs have shown to be able to deliver drugs to be released slowly for a period to provide increased antitumour ability with decreased side effects. A few PNPs are in various clinical trial stages, and some have been approved for clinical use, such as poly(lactide-*co*-glycolide (PLGA) and PLGA-polyethylene glycol (PEG), for treatments of prostate and ovarian cancer respectively (*Table 1*).

PNPs	Nanoparticles Preparation Method	Drug	Targeted Ligand	Cellular Target	Cancer lines	*In vitro / In vivo*	References
PLGA	Nanoprecipitation	Docetaxel	A10 aptamer	Prostate-specific membrane antigen (PSMA)	Prostate cancer (LNCaP) cells	*In vivo* in mice	(Cheng et al., 2007)
PLGA-PEG	Oil in water-solvent evaporation	Doxorubicin	Novel peptide	Epidermal growth factor receptor (EGFR)	Human ovarian cancer (SKOV3) cells	*In vitro* and *in vivo* in mice	(Lin, 2012)

Table 1 *– Polymer Nanoparticles developed for the delivery of drugs to treat various cancers.*

Devulapally & Paulmurugan, 2013

Non-viral gene delivery carriers

A range of non-viral nanovectors such as lipoplexes and polyplexes show potential as carrier systems for therapeutic delivery as they can protect cargo from unwanted degradation during the transfection process. Research conducted into polyplexes and lipoplexes exhibit distinct cellular uptake mechanisms in cell lines (A549 pneumocytes and HeLa cells) (Pandey & Sawant, 2016).

Polyplexes

Delivery of therapeutic nucleic acids to their site of action is a current limitation in the sector, and a combination of nucleic acids with polymeric carriers to produce polyplex nanoparticles may be a delivery method to this problem (Lächelt & Wagner, 2015). Polyplexes (PPs) are interpolyelectrolyte complexes in which genes or siRNA are complexed through electrostatic condensation between cationic groups of a polymer and negatively charged nucleic acids (Hess et al., 2017). In the formulation of PPs, cationic polymers are typically used due their ability to cooperate easily and bind nucleic acids (Vasiliu et al., 2017). They can defend nucleic acids from enzymatic degradation and facilitate cargo delivery to tumour sites (Joshi et al., 2015), which is a promising approach in anticancer strategies. For efficient intracellular delivery, several obstacles need to be conquered by the polymer-based nucleic acid carriers (*Figure 9*).

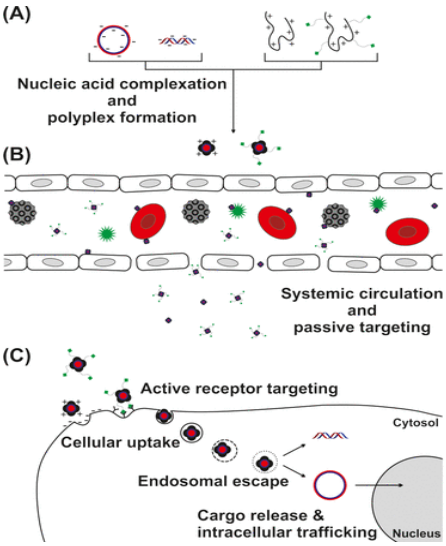


Figure 9 *- Barriers in the nucleic acid delivery pathway of polyplexes. (A) Formation of stable polyplexes, (B) avoidance of rapid clearance and unspecific interactions with blood components, and (C) cellular barriers*

Lächelt & Wagner, 2015

A carrier that when coupled with siRNAs to form polyplexes is the highly regarded polycationic transfectant polyethyleneimine (PEI). The structure of PEI constitutes of amino nitrogen and repeating units of two aliphatic carbon groups and can be found in both branch and linear morphologies with molecular weights of 200-1,500 kDa, with the latter having more favourable properties (Hall et al., 2017). Gene delivery using PEI constitutes of condensation

of RNA into dense molecules, cell uptake, endosomal escape into the cytoplasm, and uptake into the nucleus. The combination of polyplexes and PEI lead to RNA protection, intracellular release and good cellular delivery (Kim et al., 2016), however PEI's high transfecting efficiency can come with small cytotoxic complications and safety concerns, therefore slightly hindering their development as therapeutic polycationic nucleic acid carriers. This limitation is being researched, with suggestions of a modified or lower molecular weight version of PEI to decrease toxicity whilst upholding delivery efficacy being explored.

A class of cationic polymers that show high potential developed explicitly for nucleic acid delivery are poly(β-amino esters) (PBAEs), which are synthesised by joining amino acids to diacrylates (*Figure 10*). They can effectively condense nucleic acids into polyplexes that can be internalised into cells due to the polymers positive charge (Karlsson et al., 2020). An advantage of using PBAEs is their structural flexibility, which allows for easy alterations to enhance their delivery assets and decrease cytotoxicity.

Furthermore, their ability to release their cargo into specific cellular compartments via numerous degradation mechanisms, alongside exhibiting high transfection ability, good cellular uptake and high endosomal escape displays their attributes as a highly regarded delivery vector (Núria Puigmal et al., 2023).

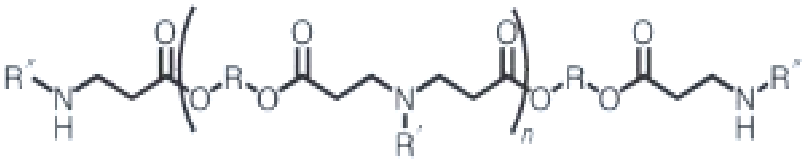


Figure 10– *The chemical structure of PBAE (Karlsson et al., 2020)*

Lipoplexes

Lipoplexes are complexes formed between lipid-based delivery vehicles and nucleic acids, typically used in cancer and cystic fibrosis studies. They are formed by the electrostatic binding of cationic lipids to negatively charged nucleic acids, with the most known lipoplex being Lipofectamine™ RNAiMAX transfection reagent. Lipoplex nanoparticles use amphiphilic lipids to hold hydrophilic therapeutics, and the cellular uptake is observed by revealing cells to

lipoplex nanoparticles in medium (Rafael *et al.,* 2015). Endocytic pathways concerning endosomes and lysosomes internalise the lipoplexes to cells. Studies show the release of drugs, such as siRNA, into endosomes and lysosomes is frequently found after 24 hours (Wu et al., 2011). The slow endosomal escape shows to be a limitation in intracellular delivery and limits the effectiveness of lipoplex nanoparticles as therapeutic applications (Boukany et al., 2013).

Furthermore, a major hurdle is when cationic lipids are combined with nucleic acid cargos, they may stimulate a strong inflammatory response in the host (Zhdanov, Podobed and Vlassov, 2002). This immune response can reduce the efficiency of the therapeutics with unwanted side effects. Therefore, lipoplexes need to show higher transfection efficiency, more effective endosomal escape, and a lower immune response to be considered as therapeutic delivery vehicle candidate.

Cell penetrating peptides

Another delivery system researched for cargo delivery is the use of cell penetrating peptides (CPPs), which are a collection of peptides that can cross the cell membrane without the aid of specific receptors, making them ideal for the intracellular delivery of many different cargos, such as proteins, nucleic acids and nanoparticles. Conjugation of pharmaceutical agents to CPPs could enhance their function and cellular uptake (Sadeghian et al., 2022). CPPs with 5-30 amino acids can supply a range of biomolecules into cells, such as siRNA, proteins and vaccines, as well as delivery of chemotherapeutic agents (Kersemans & Cornelissen, 2010 ; Wang et al., 2014 ; Guo et al., 2016).

The use of CPPs is particularly useful in the delivery of large macromolecules, such as nucleic acids. A multitude of diseases and cancers use siRNA for gene silencing, and CPPs are able to improve cellular uptake as they can overcome the poor permeability barrier and internalise the siRNA in a non-cytotoxic manner (Eguchi & Dowdy, 2009). An example of a successful CPP conjugate is the porphyrin antiviral drug CPP conjugate which can cross the blood-brain barrier, subsequentially inhibiting brain-resident HIV virus causing HIV-associated neurocognitive disorders (HAND) *in vitro* (Torchilin, 2008).

The exact mechanism of a CPP is dependent on the cargo, membrane composition and cell type, however the mechanism of penetration of CPPS is still vague (Khan, Filipczak and Torchilin, 2021). Whilst various pre-clinical studies may show promise for CPP-mediated delivery in a variety of disease models, further investigations into the tolerability and toxicity of CPPs is necessary to guarantee biocompatibility.

Methods to overcome limitations

Producing useful delivery systems to overcome the main challenges of nucleic acid cargo transport, protecting against degradation, and facilitating release at a desired location is important for treatments for a range of diseases.

Micelleplexes

An emerging solution to overcome these barriers is the use of precision medicine, where the nanomaterial design can be precise in terms of size, shape and dispersity of material to rigidity, surface chemistry and localisation of function (Street et al., 2022). Many nonviral delivery systems have been researched to overcome these limitations, with polymeric systems being the most accepted. They can be modified and are versatile, producing materials of in many shapes and sizes however, they have low transfection efficiency and stability remains a problem. Regardless, many polymeric delivery systems have been created, with the poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) being the most widely researched. This system can complex nucleic acids through electrostatic exchanges, whilst allowing for endosomal escape and cellular internalisation (Street et al., 2022). The complexation process leads to the creation of polyplexes made of an ionic core of a cationic polymer and an anionic nucleic acid, alongside a cationic polymer surface (Street et al., 2023; Zhang and Wagner, 2017). This leads to the destabilization of endosomes, which contributes to transfection efficiency (Thapa & Narain, 2016).

However, due to the formation being based on kinetic control, the shape, size and stability remains a challenge to regulate. Several other methods have been researched into efficient nucleic acid delivery, such as polymer micelles, also known as micelleplexes (Pereira-Silva et al., 2020). These are nucleic acid polymer complexes where the nucleic acid cargo is

compounded to the polymer micelle, as opposed to polyplexes where it is complexed within the particle core. They ensure effective cargo protection and transportation, alongside improved cellular transfection. Studies show that PDMAEMA polyplexes bound to the micelle exhibit higher transfection activity in contrast to spherical PDMAEMA, however further studies are required to examine whether micelles as efficient nucleic acid delivery vehicles are applicable for nucleic cargo delivery (Jiang, Lodge and Reineke, 2018; Tan et al., 2019).

An example of successfully using micelleplexes to eventually be able to deliver nucleic acid cargo can be found in a treatment of osteosarcoma (OS). This is a rare and aggressive bone cancer, with low survival rates and high relapse and metastasis occurrence (Melim et al., 2020). A study was conducted into the use of invertible micelleplexes, which are formed by changing the environmental polarity. Results showed that synthesizing the copolymer PEG-PMAN to micelleplexes demonstrated cytotoxicity against OS cells, which inhibited their proliferation. Furthermore, the micelleplexes allowed for directed transportation of the treatment to cancer cells and due to their small size and shape being controllable, a higher drug load capacity was possible (Kesharwani et al., 2018).

Proton sponge effect

In gene therapy, the ineffective delivery of siRNA to the cytosol by polyplexes or lipoplexes is usually due to the degradation of the siRNA in the early endosomal compartments (Wojnilowicz et al., 2018). Reports show that only an estimated 0.01-2% of siRNAs are able to break out of the endosome to reach the cytosol (Gilleron et al., 2013; Dowdy, 2017). Theories into whether polyplexes that display a high proton buffering capacity release their nucleic acid load from the endosome due to the “proton sponge effect” have been proposed by researchers in recent years (Rehman, Hoekstra and Zuhorn, 2013).

The concept of the proton sponge effect is not a widely accepted theory currently and requires further investigation; however, the theory is still taken into consideration as a possible method for successful endosomal escape.

The concept starts with the cellular uptake of polyplexes by endocytosis, where the inner endosomal compartment is acidified due to the adenosine triphosphate (ATP)-dependent proton pump. Subsequently, the inner pH levels of the vesicles change in the early endosomes from ~7.2 to ~6.3 and to ~5.5 in late endosomes. Due to the pH change, a large influx of protons and chloride are triggered, resulting in an osmotic balance which causes an influx of water to the endosomes. Consequently, osmotic swelling occurs and ruptures the endosome, leading to the release of the nucleic acid cargo to the cytosol.

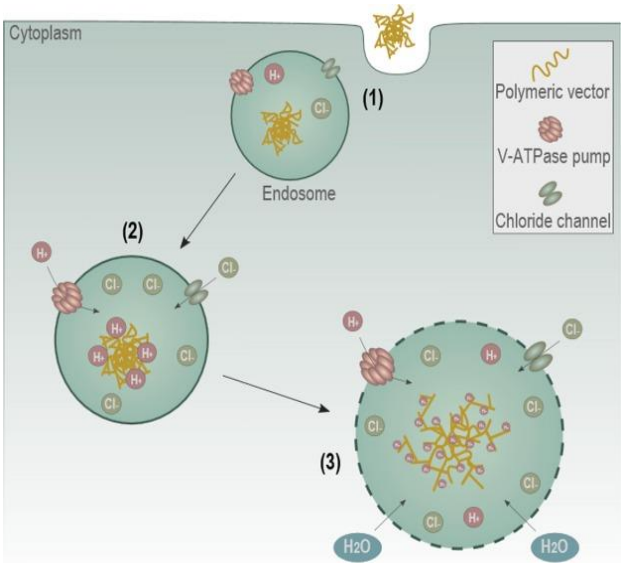


Figure 11 *– A representation of the proton sponge hypothesis according to Behr and colleagues.* ***(1)*** *Polyplexes reside in endosomal vesicles after entry to cells through endocytosis.* ***(2)*** *Upon maturation, the ATP proton pumps actively translocate protons into the endosomal lumen. The high buffer capacity polymers can bind to the protons, limiting acidification of the endosome.* ***(3)*** *The proton pump will not translocate more protons to the endosomal compartment to try to lower the pH. This is accompanied by the entry of chloride ions, leading to an increase in ionic concentration. Osmotic pressure and swelling lead to endosomal rupture, release the content into the cytosol (Behr, 1997).*

Debates occur in literature into how responsible the proton sponge effect (*Figure 11*) might be for translocation of endocytosed molecules to the cytosol (Boussif et al., 1995; Benjaminsen et al., 2013; Godbey et al., 2000; Akinc et al., 2005). During the 90's, researchers learned that various cationic polymers with extensive buffering capacities under physiological pH (e.g., lipopolyamines) could facilitate high transfection effectiveness without needing membrane-disruptive agents to be added (Haensler and Szoka, 1993). These findings inspired Bousiff *et al*. in 1995 to start research the gene delivery potential of polyethylenimene (PEI) (*Figure 12 A&B*), where it has since been a topic of interest in the research sector (Vermeulen et al., 2018).

Theories into PEI using the proton sponge effect to deliver nucleic acids by overcoming lysosomal sequestration have been proposed, although so far, no conclusive proof has been found (Richard et al., 2013). Subsequently, an equal number of opposing studies have been conducted to disprove the hypothesis, with studies showing that change in lysosomal pH is not due to PEI, and quantification of the concentration of PEI in lysosomes is perhaps not the main mechanism of polyplex escape (Wojnilowicz et al., 2018) (Roy et al., 2020).

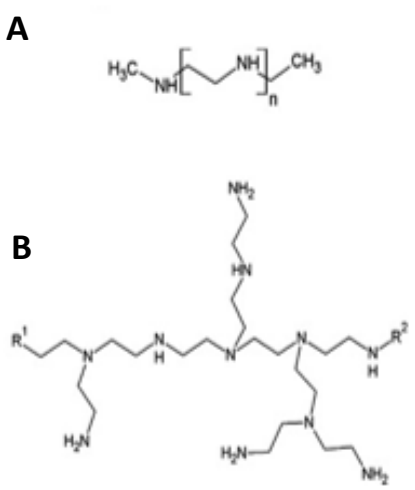


Figure 12 *- Adapted chemical structures of cationic polymers used for mediating transfection efficiency. (**A**) linear polyethylenimine (PEI) and (**B**) branched PEI.*

Concluding remarks

In summary, the research into nucleic acid delivery systems, including lipid nanoparticles, polymeric nanoparticles, and polyplexes, hold great promise for advancing the field of gene regulation and therapy. Lipoplexes and cell penetrating peptides remain systems that show potential for successful cargo delivery, and further exploration into their use as delivery vehicles required. These delivery systems offer unique advantages in targeted delivery to specific cells, facilitating cellular uptake, protecting nucleic acid cargo, and eventually promoting endosomal escape. LNPs particularly show potential as delivery vehicles as they can efficiently encapsulate and protect nucleic acids, whilst providing stability and defence against degradation. Furthermore, their lipid-based composition allows for easy modifications of their characteristics, allowing for targeted delivery. The most known use of LNPs was in the Pfizer-BioNTech COVID-19 vaccine, where it was used as a delivery vehicle for mRNA.

Additionally, polyplexes offer similar advantages including high loading capacity, protection from degradation, and enhanced cellular uptake. They have shown effective delivery and have

been successfully used for the delivery of small interfering RNA alongside a few effective uses with a complexion to mRNA, with the most notable use in the influenza vaccination. Research into the proton sponge hypothesis shows interesting theories into the endosomal escape of nucleic acids whilst using polyplexes, however a substantial amount of research is required to prove the hypothesis due to numerous conflicting studies. Furthermore, research into polymeric micelles as delivery vehicles needs to be conducted, with their successful use in the treatment of osteosarcoma providing a basis into future studies.

Whilst polyplexes remains useful delivery systems, lipid nanoparticles remain the most widely used and efficient delivery system for RNA therapeutics. Research efforts continue to focus on the optimisation of LNPs and developing delivery vehicles for mRNA and ASOs delivery, aiming to further improve safety, efficacy and clinical translation of RNA therapeutics. Novel polymers are being currently explored as versatile tools for therapeutic gene delivery, as when assembled with nucleic acids to form polyplexes or nanoparticles, the limitations of endosomal escape, cellular degradation and transport into intracellular compartments can be conquered.

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The development of novel polymeric nanoparticles as efficient non-viral RNA delivery vectors

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University of Nottingham, School of Biosciences, Sutton Bonington LE12 5RD

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Abstract

In recent years, the development of mRNA-based therapeutics has emerged as a promising strategy for addressing a wide range of diseases, including genetic disorders, infectious diseases, and cancer. However, the efficient delivery of nucleic acids to the cytoplasm of cells remains a formidable challenge. Polymeric nanoparticles (PNPs) have gained significant traction as versatile and effective carriers for mRNA delivery due to their tuneable properties, biocompatibility, and ability to protect nucleic acid cargo from degradation. However, their successful application hinges on their ability to efficiently internalise through the cell membrane to the endosome, and to subsequently escape the endosome to release their nucleic cargo.

The composition of the PNPs influence their ability to overcome these challenges, and two novel polymers named TEPA-3L2 and TETA-3L2 with different nitrate-phosphate (N/P) ratios are explored in this review. Investigations using the HEK293T cell line were conducted into the endosome-disrupting compound chloroquine combined with the N/P derivates of the PNPs to observe if transfection could be improved. The Cy-5 eGFP mRNA cell reporter system was used to visualise the internalisation and transfection capabilities of the PNPs. Furthermore, investigations into inhibition of endocytosis pathways were performed to understand the uptake mechanism of TEPA-3L2 and TETA-3L2.

In transfection and cytotoxicity assays, the N/P derivatives displayed high transfection and low toxicity, however when combined with chloroquine the transfection efficacy significantly reduced, suggesting endosomal escape is not the bottleneck in the success of the PNPs. On the other hand, the endocytosis pathway inhibitor studies displayed that the N/P ratios of TEPA-3L2 were able to successfully reduce transfection and prevent co-localisation of mRNA. Initial uncertainty into whether decreased transfection was due to cytotoxic effects were eventually overcome and the role of the clathrin-dependent endocytosis pathway in the uptake of TEPA-3L2 became evident. The experimental findings shed light on the current issues of effective mRNA transfection of TEPA-3L2 and TETA-3L2, which can guide their development towards achieving higher efficacy and lower cytotoxicity, resulting in optimal transfection.

Contents

1. Introduction

The concept of using ribonucleic acid (RNA) as a therapeutic agent has been explored since the 1980s, however only in recent years has it been considered as a potential for therapeutic treatment of disease. Currently, conventional drug strategy consists of using small molecule drugs to target the active sites of proteins to alter or inhibit their function (Damase et al., 2021), however research displays that only ~1.5% of the human genome encodes proteins (Ezkurdia et al., 2014), and only 10-14% of those proteins have active binding sites that can be targeted (Hopkins and Groom, 2002), requiring high specificity in therapeutics.

The key differences between the success of small molecule drugs and RNA-based therapeutics are due to their mode of action, targeted molecules and cell penetration capability. Small molecule drugs mainly refer to chemically synthesised compounds with a low molecular weight of <900 kDa (Li and Kang, 2020), whilst polymeric RNA varies from 7-20 kDa (Dowdy, 2023), allowing for small molecule drugs to have easier administration routes. Small molecule drugs exhibit varying degrees of molecule interaction specificity, which is not ideal for precise drug delivery, whilst RNA therapeutics offer a high degree of accuracy and specificity as they can be designed to target specific gene sequences, permitting intervention at a genetic level (Mollica et al., 2022). Nevertheless, the use of small molecules in current treatments of diseases is primarily due to its cell penetration mechanisms, as target sites can be reached easily after membrane penetration, which is the limiting factor of the macromolecule RNA (Gallego and Varani, 2001).

However, RNA-based therapeutics offer unique advantages over traditional small molecule strategies, with its usage gaining attention and momentum in recent years. The primary advantages comprise of precision and specificity, as small molecule drugs have varying degrees of specificity, potentially leading to off-target effects (Paunovska, Loughrey and Dahlman, 2022). Alternatively, specific types of RNA such as small interfering RNAs (siRNA) and antisense oligonucleotides (ASOs) can be designed to target specific genes with high precision, which minimises impact on non-diseased cells (Zhu et al., 2022). RNA therapeutics can be designed to target specific gene variations or mutations, making them favourable for treatments of rare genetic disorders. Furthermore, novel modalities can be introduced for drug

delivery, such as messenger RNA (mRNA), to instruct cells to produce specific proteins to replace malfunctioning or missing proteins.

Further research into RNA-based therapeutics was enhanced after the success of the Pfizer-BioNTech and Moderna mRNA vaccines against COVID-19, resulting in many diseases exploring the use of RNA for treatments, such as cancer, infectious diseases and genetic disorders. RNA based therapeutics offer unique advantages, specifically their ability to target gene expression at a genetic level, and research into overcoming challenges accompanying RNA delivery and stability are being explored.

Currently, the main challenge posed in the development and use of RNA in therapeutics is the ability to deliver negatively charged RNA across the cell's hydrophobic membrane. Targeting intracellular proteins tends to be challenging, as to reach the cell's cytosol where the therapeutic can be beneficial, the exogenous molecules need to surpass the membrane, as opposed to small molecules which passively diffuse across the lipid bilayer (Dowdy, 2023). Due to the large size and high negative charge density, the RNA exhibits low cellular uptake efficiency (Wang et al., 2018). Consequently, the RNA requires an efficient delivery vehicle to surpass the cell membrane to allow for interactions in the cell. The mRNA COVID-19 vaccine utilised a delivery vehicle called a lipid nanoparticle (LNP) to facilitate entry of the mRNA into cells by crossing the cell membrane, allowing for the cells to produce the crucial spike protein of the SARS-CoV-2 virus (Wilson and Geetha, 2022). Whilst LNPs hold high potential for RNA therapeutic delivery, polymeric nanoparticles (PNPs) offer advantages where LNPs may not be suitable, such as higher optimisation, targeted and controlled release of therapeutics, and tunable properties of their structure, composition and surface properties.

PNPs are sub-micron (1 to 1000nm) colloidal particles (Mehanna, Mohyeldin and Elgindy, 2014), synthesised in a nanosphere or nano-capsular shape and structure (Bhasarkar and Dharmendra Kumar Bal, 2021) (*Figure 1*). Nanoparticles can improve the stability and dispersion of cargos, help transportation across the cell membrane, and facilitate the cytosolic delivery of RNA to cells (Jiang, Abedi and Shi, 2021). These polymers can be divided into two categories: cationic and non-cationic polymers, depending on their physiological charges. The most frequently used type in therapeutic delivery are cationic polymers due to their ability to complex with anionic RNA molecules through electrostatic interactions, enabling the delivery of RNA. Whilst cationic polymers are often cytotoxic, modifications of the polymer properties such as molecular weight and charge density can resolve the issue. The most widely studied cationic polymer for gene delivery is Polyethyleneimine (PEI), which is highly efficient in non-viral gene transfection however its transfection potential is accompanied by cytotoxic effects due to its high cationic charge density and nonbiodegradability, preventing its use in clinical practise (Spain et al., 2011; Lungwitz et al., 2005; Liu et al., 2022).

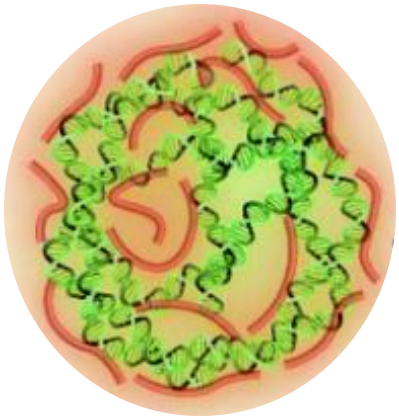

Figure 1 – Schematic illustration of a polymeric nanoparticle (Riera et al., 2019)

PNPs undergo endocytosis, a cellular process where cells internalise molecules from the extracellular environment. There are four types of endocytosis; pinocytosis, phagocytosis, caveolae-dependent and receptor-mediated endocytosis, also known as clathrin-mediated (CME), which is the best characterised process. CME consists of the formation of vesicles that enclose the nanoparticles, surpass the plasma membrane and enter the cell (Rennick, Johnston and Parton, 2021). The PNPs are designed with surface properties that allow for interactions with the receptors or proteins on the cell membrane,

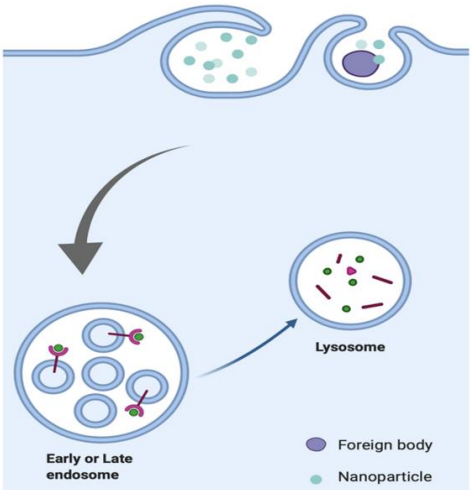

Figure 2 – Schematic representation of a polymeric nanoparticle undergoing endocytosis and maturation of the endosome into a lysosome (Niaz, Forbes and Raimi-Abraham, 2022).

such as hydrophobicity, charge and functional groups, initiating endocytosis (*Figure 2*). The cell membrane then folds inwards with the PNP attached, forming a clathrin-coated vesicle (CVV) which fuse with early endosomes, which are small compartments that move further into the cell through variety of cellular processes, and deliver its cargo (Iversen, Skotland and Sandvig, 2011). The endosomes eventually mature into lysosomes, where their pH decreases, creating an acidic environment. The decreasing pH triggers activation of enzymes such as proteases or nucleases, which eventually degrade the nucleic cargo, making it unusable.

PNPs offer several advantages as vectors for transport of RNA across the cell membrane, such as protection of the nucleic cargo, targeted delivery and sustained release (Devulapally and Paulmurugan, 2013). The nucleic cargo is susceptible to enzymatic degradation in the extracellular environment, and PNPs provide a protective shield which maintains the stability of the RNA (Pudlarz and Szemraj, 2018). Additionally, surface modifications of the PNPs can allow for targeted delivery to tissues or cells, providing an advantage over current small molecule therapies, alongside the ability of PNPs to provide controlled release of the RNA to maintain the therapeutic benefits over an extended of time (Mitchell et al., 2020). Whilst PNPs offer these advantages, ultimately the bottleneck of the success of RNA therapeutics is once the RNA has been assembled with a PNP to surpass the cellular membrane, it needs to undergo endosomal escape into the cytosol where the nucleic acids can be translated into proteins (Wang et al., 2018). Successful endosomal escape can improve the potential of nucleic acid therapies, however it remains a major hurdle. The development of effective nucleic acid systems is an ongoing area of study, with new strategies such as engineered nanoparticles being continuously explored to improve the efficiency, biosafety and specificity.

Tailored chemical synthesis of nanoparticles plays a key role in the development of innovative materials for clinical applications. Subtle chemical and structural changes can impact the biological properties of PNPs; therefore, the polymer composition and synthetic methods must be designed to be effective and nontoxic, which remains a challenge to achieve (Sathya Srinivasachari et al., 2006). Consequently, devising new synthetic methods to produce biocompatible polymers that are tailored to their specific function is key to therapeutic success. Recent experiments in the field suggest that derivatives of the chemical compounds Tetraethylenepentamine (TEPA) and Triethylenetetramine (TETA) may be suitable for RNA gene delivery, with studies conducted into the success of transporting siRNA through the use

of TEPA-modified polymers (Liu et al., 2022; Endiries Yibru Hanurry et al., 2020) or TETA-modified polymers (Hoon Jeong et al., 2007; Kim et al., 2012). In therapeutics, siRNA is used for specific functions, such as silencing or reducing the expression of genes, however mRNA has different functions such as to stimulate the immune system or produce new proteins. Subsequently, research is required to establish the full potential of PNPs transporting mRNA successfully to the cytosol, as there is currently no research on this design system.

TEPA ($C_8H_{23}N_5$) and TETA ($C_6H_{18}N_4$) are chemical compounds with derivatives that have been explored for their potential use in nucleic acid delivery (*Figure 3*). Whilst they are related compounds, TEPA contains four ethylenediamine units linked by methylene bridges and has a molecular weight of 189.3 Da, whilst TETA contains three ethylenediamine units and has a molecular weight of 435.2 Da. They are positively charged polyamine compounds with several amine groups that can interact with negatively charged RNA and traverse the negatively charged cell membrane, facilitating entry to endosomes (van Dam, 2002).

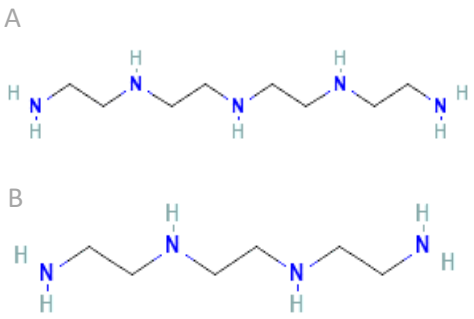

Figure 3 – Chemical structures of TEPA [A] and TETA [B] (PubChem).

Due to their multiple amine groups, both TEPA and TETA may be functionalised in many ways. One possibility is an attachment of functional groups which may serve as ligands for host-guest interactions with cucurbit[8]uril (CB[8]), which is a macrocyclic molecule composed of eight glycoluril units linked by methylene bridges (Sun et al., 2023). Through non-covalent interactions, mostly hydrogen bonding and hydrophobic interactions, the CB8 encapsulates two guest groups (Qiao et al., 2017). A low molecular mass compound containing two such guest groups forms a supramolecular polymer with CB[8] through a host-guest interaction. The polymers explored in this study each have three CB[8] guest-groups, forming branched polymeric molecules which have greater potential as delivery vectors than their linear analogues (Ahmed and Narain, 2012; Zhou et al., 2016). Due to their guest groups, the polymers are termed TEPA-3L2 and TETA-3L2.

The nitrate to phosphate ratio (N/P) is considered an important physiochemical property of polymeric nanoparticles. The ratio of positively charged polymer amine (N) groups to

negatively charged nucleic acid phosphate (P) groups of a polyplex influences its properties such as the size, stability and surface charge. Discovering the ideal N/P ratio for polyplexes requires optimisation and experimentation to observe the cytotoxic effects, biocompatibility and transfection efficiency that occurs. Consequently, four N/P ratios have been used for experimentation for the polymers TEPA-3L2 and TETA-3L2.

To ensure the success of PNPs in nucleic cargo delivery, methods of determining whether the RNA has escaped the endosome need to be developed to quantify escape efficiency. Engineering nanoparticles to overcome endosomal escape requires an understanding of how nanoparticles interact in our cells and their cytotoxic effects, which can be investigated through transfection, toxicity and imaging experiments. This paper examines research conducted into characterising and testing N/P ratios of the two supramolecular complexes TEPA-3L2 and TETA-3L2 for the efficient intracellular delivery of exogenous RNA, and to investigate if the challenges of endosomal escape can be overcome with these novel non-viral vectors.

2. Materials and Methods

2.1. Cell culture

Wild Type human embryonic kidney cells (wt HEK293Ts), modified wtHEK cells (mCherry-GAL9 reporter expression cells), and adenocarcinoma cells (A549's) were grown in Dulbecco's Modified Eagle's Medium (RNBL7919, Sigma) modified with 10% foetal bovine serum (Sigma, F7524) and L-glutamine (Sigma, G7513) in a cell culture flask and maintained in an incubator at 37°C and 5% $C0_2$ until they were ~80% confluent.

HEK293T's were primarily used in transfection, toxicity and imaging experiments, with mCherry-GAL9 cells used typically to observe endosomal damage or the RFP channel on imaging systems, and human lung carcinoma A549 cells used once for cell line variability. HEK293T and mCherry-GAL9 cell lines were provided by Dr Sal Jones, and A549's kindly provided by Anna Grabowska's group.

Cell lines were passaged regularly using phosphate buffered saline (Oxoid, BR0014G) for washes, trypsin (Sigma, T3924), and Dulbecco's modified eagle medium (DMEM)

supplemented with foetal bovine serum (FBS) and l-glutamine, at a passage ratio of 1:20 or less depending on confluence at time of passage. Incubation was at 37°C and 5% CO_2.

When seeding for experiments, cells were counted using a manual haemocytometer and excluding dead cells using trypan blue (Sigma, T8154). The number was averaged, and the dilution factor accounted for, followed by making a stock cell suspension comprised of cell culture and DMEM, which was seeded via multichannel to 96-well plates. Phosphate buffer saline (PBS) was pipetted to the outer cells of the plate for ensured sterility.

2.2. Polymer and RNA stocks

Concentrated polymer stocks of Tetraethylenepentamine (TEPA) with 3 CB[8] guest groups and Triethylenetetramine (TETA) with 3 CB[8] groups alongside mRNA stocks, were provided by Dr Rafał Kopiasz, Research Fellow at the Faculty of Science. The polymers are referred to as TEPA-3L2 and TETA-3L2 as internal acronyms for the 3 CB[8] guest groups.

Each formulation was produced by mixing 2x concentration polymer with 2x concentration mRNA in equal volumes. All dilutions were in Invitrogen Ambion™ DEPC-Treated Water (Nuclease-Free water). RNA concentration for polymers were 5µg/mL for assays and 20µg/mL for imaging. Final mRNA concentration depended on the N/P ratio used and the number of conditions in experiments. Final polymer solution was prepared for N/P 16 using a 1:49 ratio of polymer stock (5mg/mL unless otherwise specified) to nuclease-free water, and serially diluted for N/P 8, N/P 4 and N/P 2, before mixing with final mRNA solution.

For luciferase and cytotoxicity assays, Luciferase mRNA (fLuc, TriLink L-7202) was used for bioluminescence, whilst for imaging experiments, a mixture of four parts eGFP mRNA (Green Fluorescent Protein, TriLink L-7601) and one part Cy5-e-GFP mRNA (CY5, TriLink L-7701) were used to allow visualisation while minimising impact on translation.

2.3. DLS and Zeta Potential

The size and zeta potential of the polymeric nanoparticles were measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments). Polyethyleneimine (PEI) was used as a positive control alongside the four N/P ratios (N/P 16, 8, 4 and 2) to observe how

the different N/P ratios would affect surface charge, size and stability. The hydrodynamic diameter and PDI were measured to observe size, whilst the zeta potential indicated the stability of the colloidal dispersions. The DLS measurements used 200µl of sample in a Malvern Panalytical DTS0012 disposable cuvette with a 173° scattering angle, whilst zeta potential samples used 800µl in a DTS1070 cuvette. Measurements were recorded using the Zetasizer software that accompanied the machine.

2.4. Imaging

General fluorescence microscopy occurred on the Thermofisher EVOS M5000 unless stated otherwise. Images of cells in 96-well plates were captured using the software accompanying the machine.

Confocal imaging was conducted on the Leica SP8 Confocal Microscope. Conditions were seeded and treated in a circular quarter well cell culture dish (Greiner, Cellview 627870). The software LAS X (Leica) was used to set the excitation and emission spectra and capture images.

ZEISS Celldiscoverer 7 confocal machine (CD7) was used for imaging overnight 96-well plates. Prior to imaging, 1µl of a 1mg/mL aliquot of Hoechst 33342 was added to each well. Images were captured using the ZEISS ZEN 3.8 software at multiple time intervals.

2.5. Transfection and Toxicity Assays

Transfection and toxicity experiments used the selected N/P ratios for polymers, and each experiment had a selection of different controls; the positive controls being Lipofectamine™ MessangerMAX™ Reagent (Invitrogen), and PEI, and the negative controls being free mRNA and Opti-MEM. Two white 96-well plates were seeded in each experiment to be run separately.

A 96-well plate was seeded with 25,000 HEK293T cells per well on Day 1, formulations of the N/P ratios and controls prepared and treated onto cells on Day 2, and transfection and toxicity experiments conducted on Day 3. The transfection experiment used ONE-Glo™ Luciferase Assay System mixed equally with Opti-MEM, where all the media was removed off the top of the wells and 200µl of the luciferase solution gently pipetted to the wells. The plate was

incubated for 10 minutes before a plate read on a TECAN Spark 10M, where information about the reading light units (RLU's) were produced.

The free mRNA condition was replaced by a 'killed' condition on toxicity plates, where the treatment on Day 2 consisted of a 1:9 ratio of sodium acetate with 10% Triton X-100 (TX100) to Opti-MEM. The toxicity experiment used a 1:9 ratio of PrestoBlue™ Cell Viability Reagent and PBS to treat the cells on Day 3, where all medium was removed from wells and 100µl of the solution gently pipetted on. The plate was then incubated for 45 minutes before a plate read on a TECAN Spark 10M, where the RLU's were produced. Data was normalised against the 'killed' condition to account for variability between conditions.

2.6. Chloroquine

For experiments where cells were treated with chloroquine, 30µM of chloroquine was added to the final concentrations of the treatments of polymers and controls, and cells treated. Assays and imaging were conducted after 24 hours.

2.7. Inhibitors

Chlorpromazine and Genistein were provided in 20mg/mL and 100mg/mL aliquots respectively by Dr Robert Cavanagh, Research Fellow at the Faculty of Science. Chlorpromazine conditions used 4mg per well and Genistein conditions used 10mg per well unless otherwise specified. A mixture of one part chlorpromazine and two parts PBS was formulated as a diluted treatment. Inhibitors were spiked into wells, followed by a 30 minute incubation of the 96-well plates at 37°C and 5% CO_2. All medium was removed from the wells and formulations of the polymers and controls added, followed by a second spike of the inhibitors. Plates were then incubated for 24 hours prior to imaging and assays.

2.8. Red Fluorescent Protein tracker

CellLight™ Early Endosomes-RFP tracker (BacMam 2.0, Invitrogen) was used as directed by the manufacturer but at varying concentrations, as described in Results. Imaging was conducted on EVOS at 6, 24 and 48 hours after treatment.

3. Results & Discussion

3.1. Cell density optimisation of HEK293Ts

To find the correct density of HEK293T cells to seed for experiments, an optimisation study was conducted. Cells were seeded at 20,000 cells, 25,000 cells, and 30,000 cells per 200µl well in a clear Greiner 96-well microplate. To obtain the different densities of cells, a cell passage of the HEK293T cells was conducted and seeded at the 3 densities, each with 3 repeats. Another triplicate condition for each of the densities was seeded where cells were re-fed with Gibco Opti-MEM, a reduced serum media that can keep cells alive for 24 hours, which slowed down the growth of cells. Re-fed condition was performed to imitate what would happen when media was lifted, and therapeutic treatments were added as cell growth is disturbed. Imaging on an EVOS M5000 was conducted to observe the confluency of cells after 24 and 48 hours, and results were further applicable to the mCherry-GAL9 cell line. The density of 25,000 cells per well showed the ideal amount of cell confluency, with space for cell growth in the Opti-MEM refed conditions (*Figure 4*).

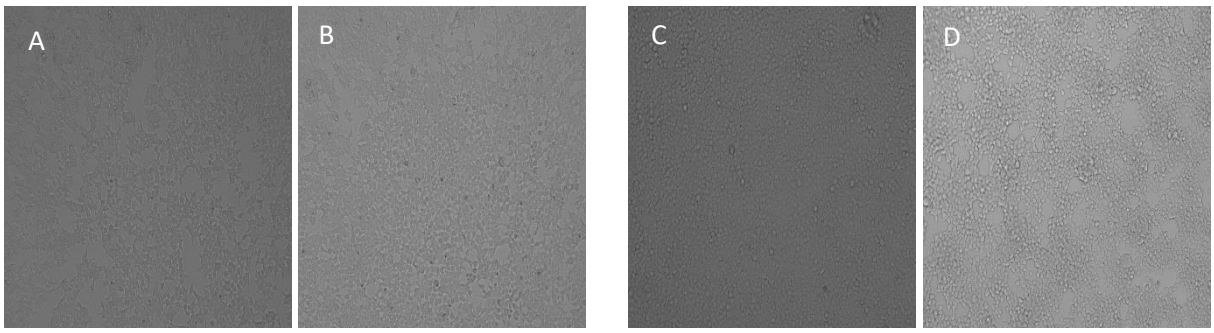

Figure 4. Seeding density of 25,000 cells per well of HEK293T cell line. Images captured on a EVOS M5000 at 24 hours (A & B) and 48 hours (C & D) after seeding. Images B & D display the Opti-MEM refed condition. Scale bar = 300 µm

3.2. DLS and Zeta Potential of TEPA-3L2 and TETA-3L2 N/P ratios

N/P ratios for TEPA-3L2 and TETA-3L2 were characterised in terms of size, polydispersity index and zeta potential to observe which ratios would be suitable for future experimentation. Zeta potential is an important parameter in establishing the colloidal stability of polyplexes, which has an effect on the cellular uptake, transfection efficacy and cytotoxicity (Shrivastava, 2018). Nanoparticles with a zeta potential between -10 and +10 mV are considered neutral, whilst between -30 mV and +30 mV are considered strongly cationic and anionic, respectively

(Clogston and Patri, 2010). Efficient interactions between positively charged polyplexes and the negatively charged cell membrane exhibit effective cellular uptake (Pack et al., 2005), however extremely high positive charges in polyplexes display cytotoxic effects, therefore decreasing the transfection efficacy (Al-Dosari and Gao, 2009).

The zeta potential values for the N/P ratios for TEPA-3L2 and TETA-3L2 can be observed in *Figure 5*. Both polymers N/P ratios of 16, 8 and 4 displayed ideal values of ~30 mV, indicating they are strongly cationic, whilst N/P 2 for both polymers displayed negative zeta potential of ~20 mV, demonstrating they are slightly anionic. The zeta potential reflects the surface charge of the colloidal particles, and a positive zeta potential is desirable due to the electrostatic interactions attracting the particle to the negatively charged cell membrane, facilitating cellular uptake.

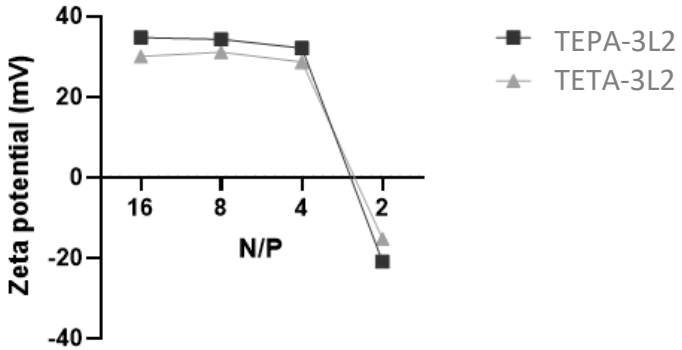

Figure 5. Zeta potential of N/P ratios 16, 8, 4 and 2 for TEPA-3L2 and TETA-3L2.

Additionally, the particle size and polydispersity index were measured for the N/P ratios of TEPA-3L2 and TETA-3L2. Particle morphology significantly impacts the efficiency of cellular uptake alongside toxicity of cells (Foroozandeh and Aziz, 2018; Nel et al., 2009). Several studies have found that an optimum particle size of ~50-60 nm has a higher uptake rate and can be internalised more efficiently. Alternatively, nanoparticle uptake displayed decreased uptake for smaller particles (~15-30 nm) and larger particles (<100 nm) (Chithrani and Chan, 2007; Geiser et al., 2005; Jin et al., 2009; Lu et al., 2009). *Figure 6* displays the hydrodynamic diameter recorded for N/P ratios 16, 8 and 4 of both polymers was ~75- 80 nm, indicating that a high cellular uptake could be possible, whilst N/P 2 for both polymers displayed a very high hydrodynamic diameter of ~200 nm for TEPA-3L2, and ~360 nm for TETA-3L2, rendering them

unsuitable. These results coincide with the zeta potential, resulting in N/P 2 being excluded from further experimentation.

The polydispersity index (PDI) was recorded for the N/P ratios using DLS to measure the molecular weight distribution. A PDI closer to 0 indicates a narrow size distribution, with particles being more uniform in size, whilst a PDI closer to 1 indicates a larger size distribution, and a wider range of particle size (Danaei et al., 2018). In polyplexes used for drug delivery, a lower PDI is desirable as it implies there is a more consistent particle distribution, leading to more predictable behaviour in cellular uptake and interactions with biological systems. The PDI of polyplexes is often considered suitable for drug delivery applications when it is around 0.3 or below (Whiteley et al., 2023). *Figure 6* shows that the PDI for TEPA-3L2 N/P 8 and 4 are the lowest, excluding N/P 2 due to previous results, with PDI's of approximately 0.45 and 0.3, respectively. Results for TETA-3L2 display similar values, with the lowest PDI's at N/P 8 and 4 of approximately 0.3 and 0.25, respectively.

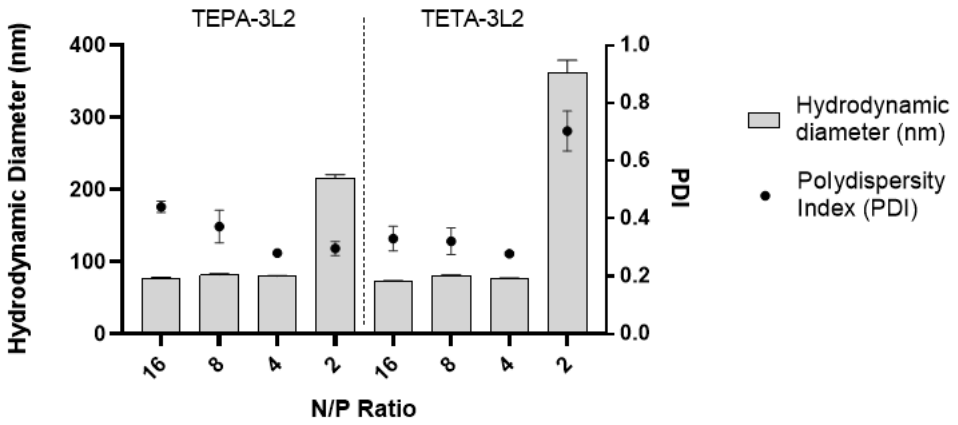

Figure 6. Hydrodynamic diameter and PDI of N/P ratios 16, 8, 4 and 2 for TEPA-3L2 and TETA-3L2.

3.3. Transfection and Toxicity Assays of TEPA-3L2 and TETA-3L2

To understand which N/P ratio of TEPA-3L2 and TETA-3L2 would work most efficiently with HEK293T cells, a transfection and toxicity test was conducted. A luciferase assay was used to determine the transcriptional activity of a target gene resulting from a delivered nucleic acid

(Carter and Shieh, 2015). The assay relies on the activity of the luciferase enzyme, which is encoded in the FLuc mRNA complexed with the PNPs. A light-emitting reaction is catalysed when the enzyme interacts with its substrate luciferin, which is present in the ONE-Glo Luciferase treatment added prior to the plate read. The emitted light from the reaction can be quantified in relative light units (RLU), providing a measure of successful transfection (Hirschenberger et al., 2021).

N/P ratios 16, 8, 4 and 2 were tested for TEPA-3L2 and TETA-3L2, alongside a set of positive controls; Lipofectamine and PEI, and negative controls; Opti-MEM and free mRNA. PEI is a polymeric nanoparticle and Lipofectamine is a lipid nanoparticle that are capable of successful transfection but are cytotoxic for clinical usage. Opti-MEM is a reduced-serum media supplemented with 10% FBS, which when treated onto cells allows for cell viability to be

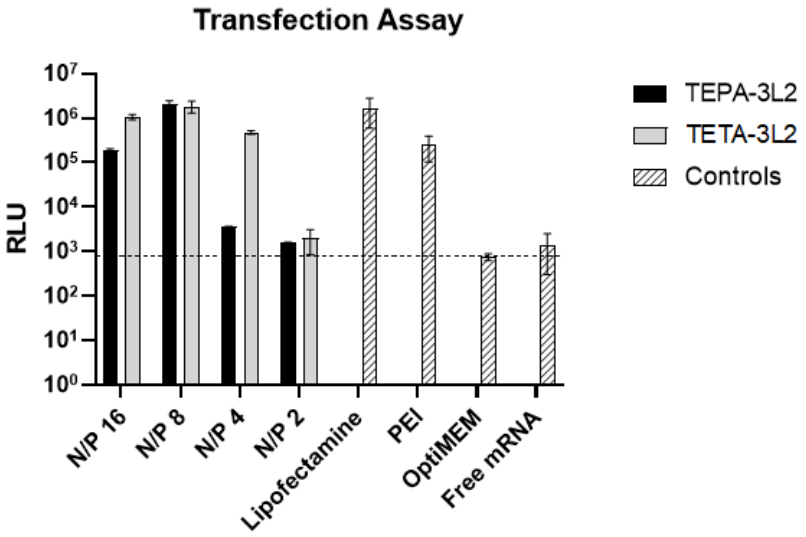

Figure 7. A luciferase assay of N/P 16, 8, 4 and 2 of TEPA-3L2 and TETA-3L2 and a set of controls.

maintained without increasing or decreasing transfection. Free mRNA serves as another negative control as it demonstrates how mRNA without a nanoparticle would act in cells, where it is known that it cannot pass the cell membrane, leading to no transfection occurring.

The Opti-MEM and mRNA conditions display the amount of RLU's produced if transfection was unsuccessful or very low, whilst Lipofectamine and PEI display the amount of RLU's produced for optimum high transfection. *Figure 7* exhibits that the N/P ratios of 16 and 8 for both polymers express high RLU's, similar to Lipofectamine, rendering them the most likely to transfect efficiently during experiments.

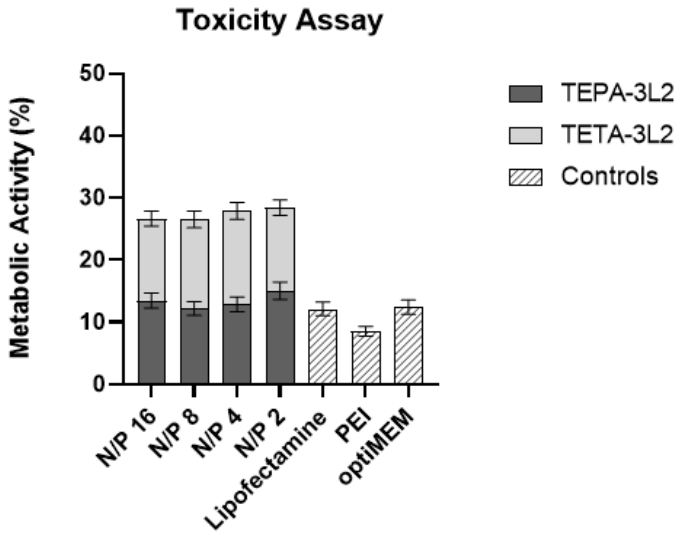

Figure 8. A cytotoxicity assay of N/P 16, 8, 4 and 2 of TEPA-3L2 and TETA-3L2 and a set of controls.

Simultaneously with the transfection experiment, a cytotoxicity assay was conducted which is used to provide insight to the potential adverse effects of substance, such as drugs, on cell viability and growth. A PrestoBlue reagent is treated onto the cells prior to the plate read to be taken up by viable cells. Higher metabolic activity suggests that the polymeric treatments or controls are not very toxic to the cells, which is ideal for drug development.

Figure 8 normalises the data against the 'killed' condition, which is a control that uses a non-ionic surfactant treatment to lyse cells. It is used as an indicator of a condition where all cells are dead, and theoretically should display the lowest metabolic activity. The metabolic activity facilitates data interpretation, allows for conclusions to be made about whether a treatment has increased or decreased metabolic activity compared to a control.

The four N/P ratios for both polymers displayed high metabolic activity, with TETA-3L2 exhibiting the highest activity. The data indicates that the polymeric nanoparticles are not toxic to the HEK293T cell line.

3.4. Timepoint experiment to observe ideal uptake time of PNPs

A time interval imaging experiment was conducted to observe at what time point does clear internalisation of CY5 and GFP mRNA occur in HEK293T cells. N/P 8 for TEPA-3L2 and TETA-3L2 was tested due to the transfection assay results, alongside N/P 4 for both polymers due to interesting results from a fellow researcher. The controls lipofectamine and free mRNA were

used. Imaging was conducted on an EVOS M5000 at 2hr, 4hr, 6hr and 24hrs after treating cells with the polymeric nanoparticles and controls (*Figure 9*).

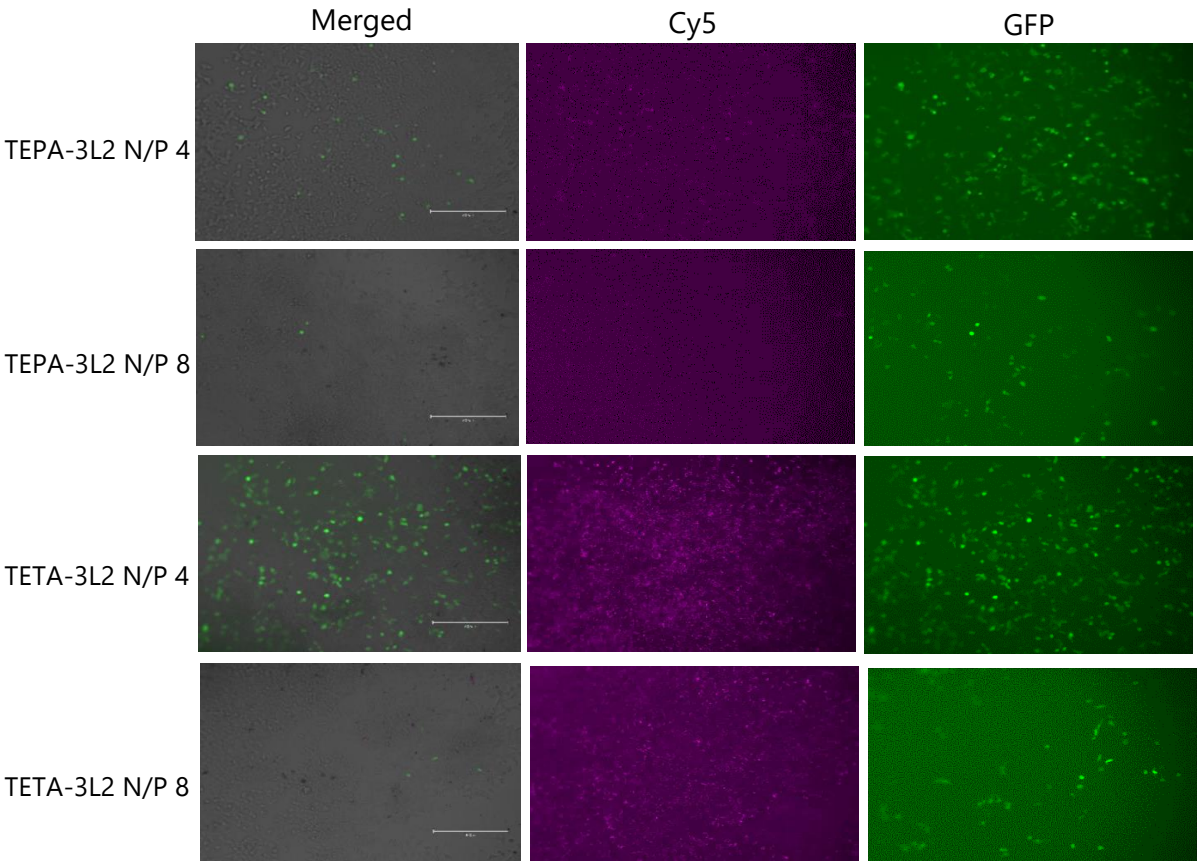

Figure 9. Uptake of CY5 and GFP mRNA after 24 hours. Channels display merged, CY5 and GFP separately for N/P 8 and 4 of TEPA-3L2 and TETA-3L2. Scale bar = 300 µm

Internalisation of the CY5 RNA encoding GFP could be seen at 24 hours (*Figure 9*), whilst the positive control lipofectamine displayed uptake at 6 hours, indicating how a different cellular uptake route and internalisation mechanism is used compared to the polymers. The free mRNA condition exhibited no uptake after 24 hours, confirming why a delivery system is crucial for successful transfection. The PNPs fluoresce pink with the CY5-labelled RNA, whilst the RNA itself encodes the green-fluorescent protein, facilitating the visualisation of successful transfection.

3.5. Observation of uptake in A549 cell line

To observe if the polymers were able to display GFP and CY5 uptake in a different cell line to kidney cells, imaging was conducted on the EVOS M5000 of the A549 lung cell line. A cell passage was conducted, and a 96-well plate seeded with 8000 cells per well and left to grow

for 24 hours. Next, TEPA-3L2 N/P 16 and 8, TETA-3L2 N/P 16 and 8, and controls Lipofectamine, PEI, Opti-MEM, and free mRNA were formulated using eGFP and CY5 RNA. The cells were then treated and placed in the incubator for 24 hours, and 1µl of 1mg/mL Hoechst 33342 was added to each well prior to imaging. *Figure 10* shows that CY5-labeled molecules could be visualised in all conditions apart from the negative controls, as expected. However, GFP expression was only apparent in lipofectamine, meaning the GFP encoding mRNA could not be transported to the appropriate subcellular compartment to be translated. Transfection may be tissue-dependent and perhaps need more optimisation in future work, so consequently A549's were excluded from further experiments.

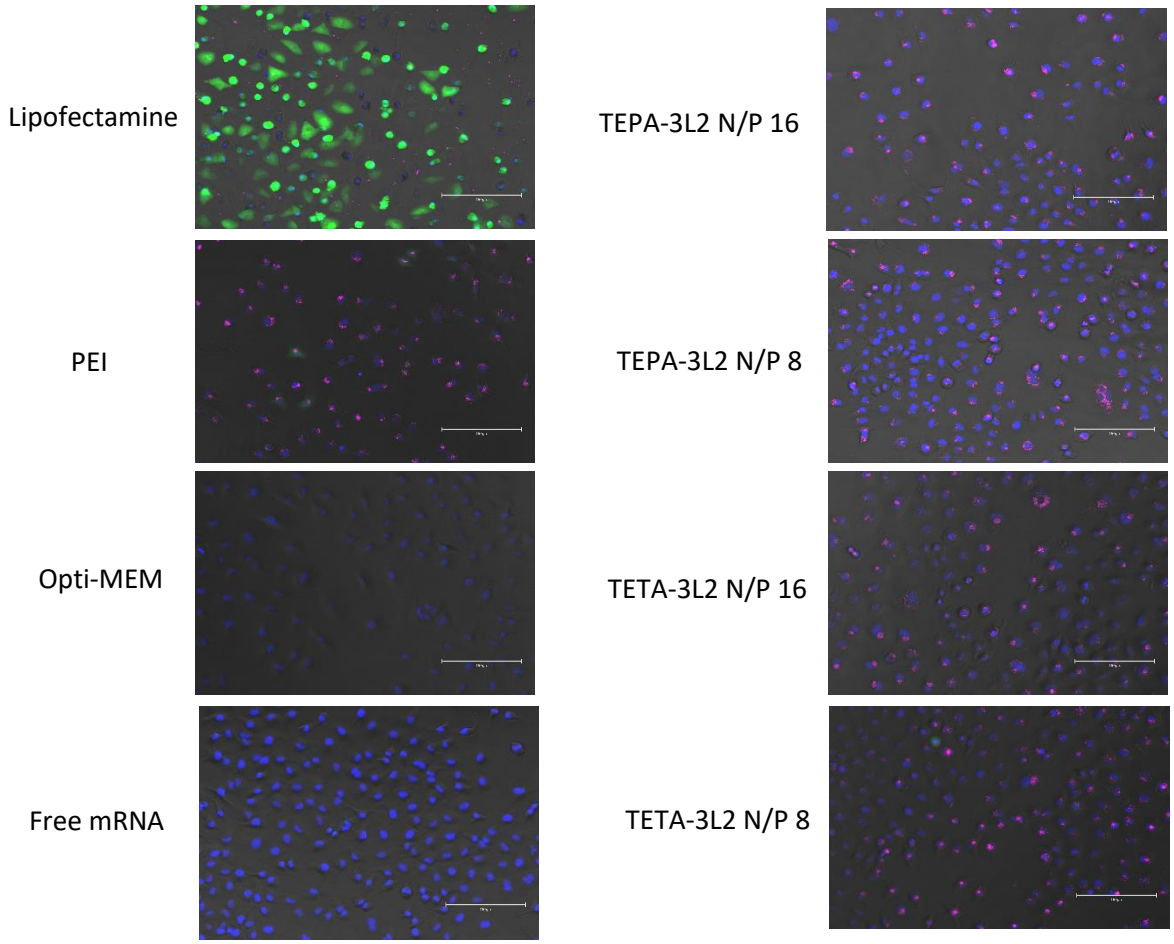

Figure 10. N/P ratios 16 and 8 for TEPA-3L2 and TETA-3L2, alongside a set of controls, were treated alongside Cy5 encoded eGFP mRNA to A549 cells to observe transfection. Scale bar = 300 µm

3.6. Effect of Chloroquine on Transfection by TEPA-3L2 and TETA-3L2

To observe if transfection could be enhanced, investigations into the use of chloroquine and the effect of increasing endosome acidification in HEK293T cells to promote endosomal escape were conducted. Chloroquine is an endosomolytic agent that has diverse effects on cells due to its ability to modulate certain cellular processes and alter endosomal pH levels. Chloroquine is commonly used as a treatment to increase the pH of endosomes in cells and study the role of endosomal compartments in cellular processes such as endocytosis (Murphy et al., 2001; Cheng et al., 2002). Several studies exhibit that chloroquine enhances the transfection efficiency of non-viral gene delivery systems; however it is accompanied by cytotoxic effects (Plank et al., 1999; Cotten et al., 1990; Zenke et al., 1990). Alternatively, a study using HEK293T cells and 100 uM of chloroquine displayed that it mediated inhibition of transfection efficiency (Wolfert and Seymour, 1998), whilst another study found that optimum transfection occurred in the absence of chloroquine (Hart et al., 1997).

Conflicting results from studies prompted an investigation, where transfection and toxicity assays of HEK293T cells were conducted, with two microclear well plates seeded and treated with formulations of TEPA-3L2 N/P 8, 4 and 2, TETA-3L2 N/P 8, 4 and 2, and controls Lipofectamine, PEI, free mRNA, and Opti-MEM. All triplicate sets of conditions were seeded in duplicate to allow for a 30 uM per well chloroquine and minus chloroquine condition.

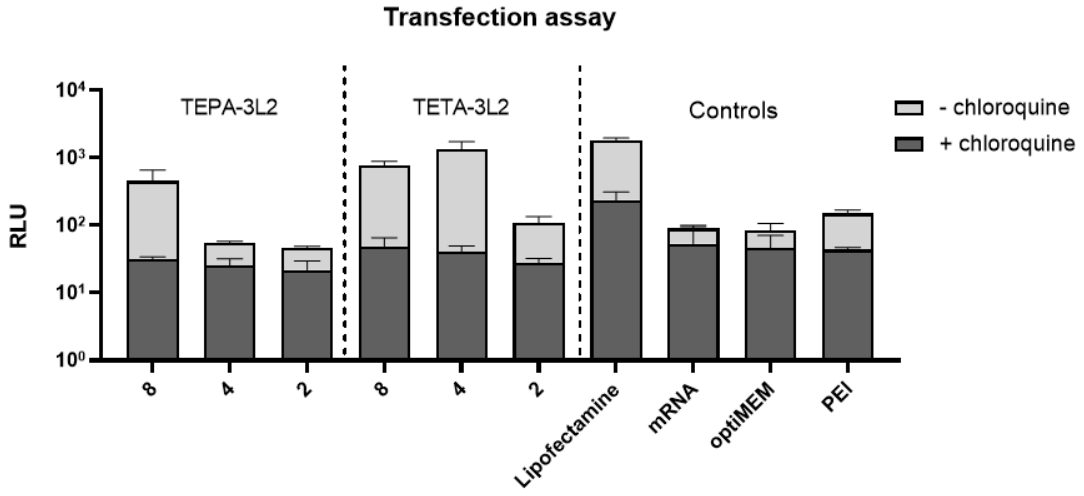

Figure 11. Chloroquine transfection assay displaying N/P ratios 8, 4 and 2 for TEPA-3L2 and TETA-3L2, alongside controls, with and without chloroquine. Conditions with chloroquine use 30 µM per well.

The results displayed in *Figure 11* demonstrates a clear reduction of transfection when chloroquine was treated to cells, with all polymeric conditions and controls transfecting higher when left untreated. These results coincide with the cytotoxicity assay data in *Figure 12*, where the metabolic activity decreases with addition of chloroquine, indicating that it is toxic to cells. Whilst it appears that chloroquine causes transfection to decrease, the RLU reduction matches the decreased metabolic activity, suggesting chloroquine has no impact on transfection itself and the reduction is likely caused by cytotoxicity.

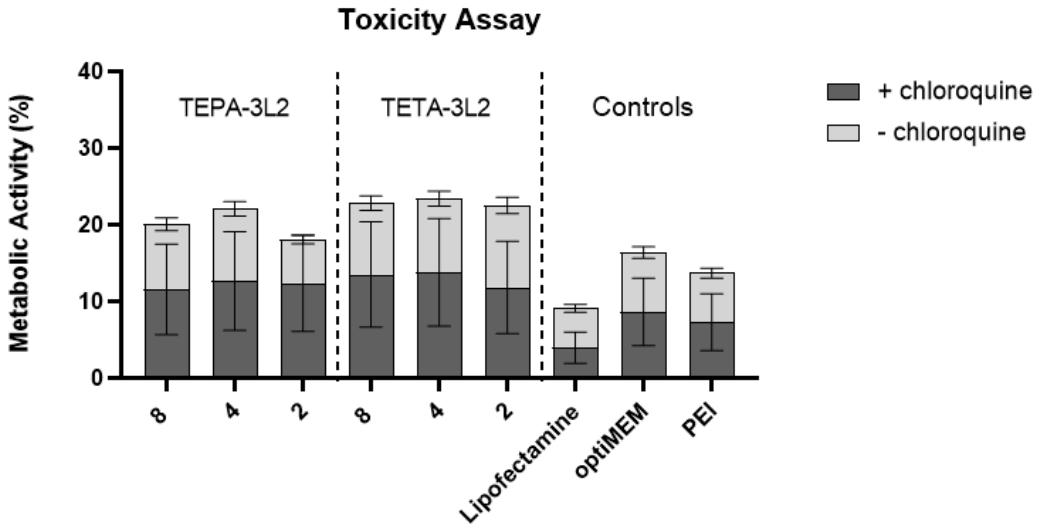

Figure 12. Chloroquine cytotoxicity assay displaying N/P ratios 8, 4 and 2 for TEPA-3L2 and TETA-3L2, alongside controls, with and without chloroquine. Conditions with chloroquine use 30 uM per well.

As the results from the previous experiment did not provide conclusive evidence into whether chloroquine has an effect on transfection, a confocal imaging experiment with chloroquine was conducted on the Leica SP8 Laser Confocal Microscope. The mCherry-GAL9 cell line was seeded to observe the red channel on the microscope. The conditions used were plus and minus chloroquine of TEPA-3L2 N/P 8, Lipofectamine and Opti-MEM, seeded on a circular quarter well cell culture dish (Greiner Cellview 627870). Each 500µl quarter well used 60uM of chloroquine, and were imaged under the GFP, CY5, and Hoechst and mCherry channels. Prior to imaging, 2.5µl of Hoechst 33342 Trihydrochloride (Invitrogen) was added to each quarter well to stain nuclei blue. The software LAS X (Leica) was used to capture images, and the excitation and emission spectra set for the channels, consisting of a 488nm excitation and 507nm detected emission for GFP, 649nm excitation and 667nm detected emission for CY5,

350nm excitation wavelength and 461nm detected emission wavelength for Hoechst, and a 587nm excitation and 610nm detected emission for mCherry.

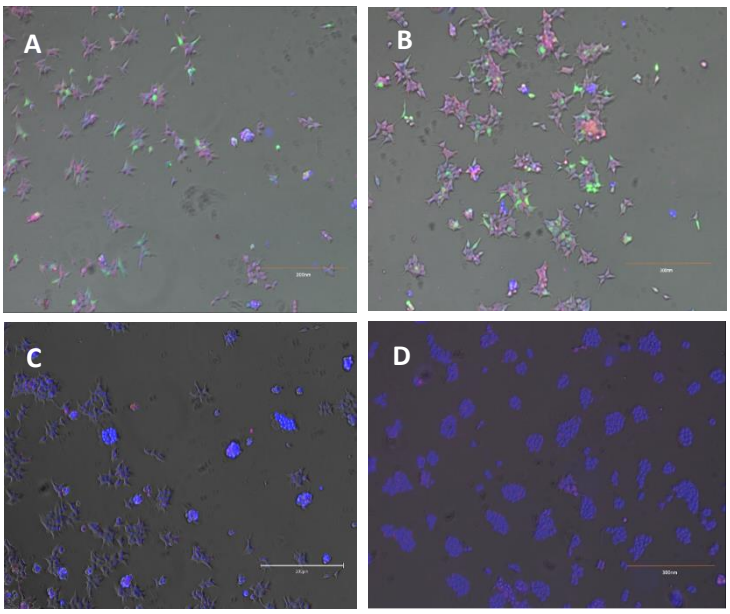

Figure 13. Confocal images of Lipofectamine with (A) and without (B) 60 µM chloroquine, and TEPA-3L2 N/P 8 with (C) and without (D) 60 µM chloroquine. Scale bar = 300 µm.

The confocal images captured did not include mCherry due to the SP8 not being able to image CY5 and mCherry channels simultaneously. *Figure 13* displays lipofectamine conditions and TEPA-3L2 N/P 8 conditions with and without chloroquine. Whilst it cannot be counted quantitatively, it is visible that the addition of chloroquine did not exhibit increased transfection of the CY5 mRNA or GFP mRNA in both conditions, particularly for N/P 8, where Hoechst 33342 is primarily visible, with faint scatters of CY5 observable. In correspondence with results from *Figure 11* and *Figure 12*, it was found that chloroquine does not enhance transfection of TEPA-3L2 or TETA-3L2 in HEK293T or mCherry-GAL9 cells. This suggests that endosomal escape is not the bottleneck of successful transfection as chloroquine has been shown to typically acidify the endosome for subsequent endosomal escape.

3.7. Effect of Uptake Pathway Inhibitors on Transfection

Further investigations were conducted into whether transfection would be affected through inhibition of endocytosis pathways. The size of nanoparticles plays a major role in determining which endocytosis uptake pathway is used (Foroozandeh and Aziz, 2018). Nanoparticles with

a size range of 120-150 nm are typically internalised through caveolin--mediated endocytosis, whilst nanoparticles ranging between 30-50 nm are typically internalised through receptor-mediated endocytosis, also known as clathrin-mediated endocytosis (Lu et al., 2009). Earlier experiments looking at the nanoparticles size established that the four N/P ratios for both TEPA-3L2 and TETA-3L2 were between approximately 80-85 nm, therefore placing them in the middle of the size range of the two types of endocytosis pathways. Investigation into the pathways of endocytosis can help identify the specific pathways polymeric nanoparticles use to deliver their cargo into endosomes, and whether transfection efficiency is reduced.

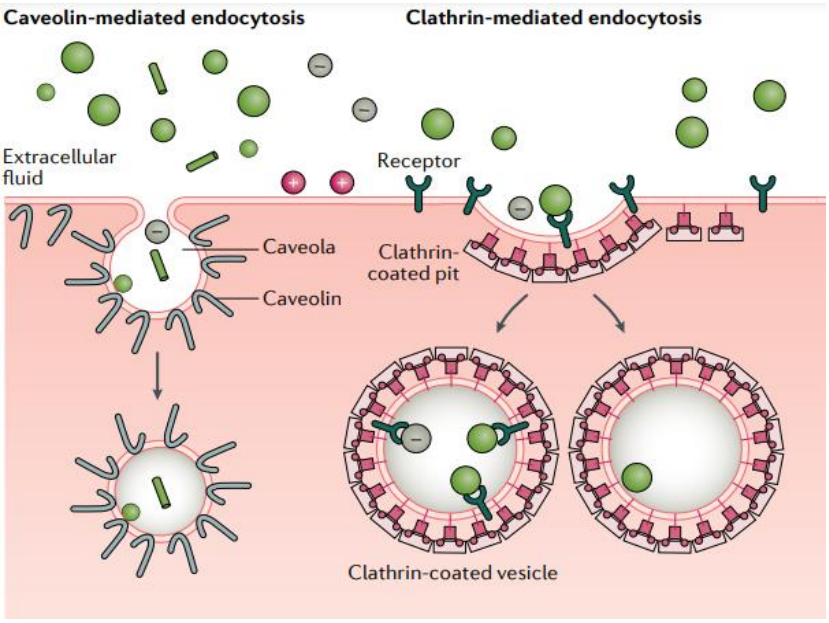

Figure 14. Clathrin- and caveolae-mediated endocytosis pathways used for nanoparticle uptake (Mitchell et al., 2020)

Chlorpromazine was explored to block the clathrin-dependent endocytosis pathway and Genistein to block the caveolae-dependent endocytosis pathway. Both inhibitors were provided in aliquots by Dr Robert Cavanagh, Research Fellow at the Faculty of Science. Chlorpromazine is a cationic amphiphilic drug believed to disrupt the assembly or inhibit the formation of the clathrin-coated pit (*Figure 14*), whilst Genistein is a tyrosine kinase inhibitor, which are enzymes that play an important role in cellular signalling necessary for the formation of caveolae (Vercauteren et al., 2010; Chang, Wu and Yuan, 2014).

The first experiment consisted of seeding HEK293T cells for a transfection and toxicity assay with the conditions TEPA-3L2 N/P 8 and N/P 4, and the controls Lipofectamine and Opti-MEM, alongside a 'killed' condition seeded for the toxicity plate. Each condition was seeded in

triplicate, with set 1 of the conditions left as a control, set 2 treated with chlorpromazine, and set 3 treated with genistein.

Prior to adding the formulations to the plates, the treatments were diluted to reduce the toxicity and reduce off-target effects. Chlorpromazine was aliquoted as 20mg/mL, so 4mg per well was required, resulting in a solution of 12µl inhibitor and 12µl PBS, allowing for two spikes of 0.4µl in each well of both transfection and toxicity plates. Genistein was aliquoted as 100mg/mL, so 20mg per well was required, resulting in a solution of 120µl of inhibitor needed, allowing for two spikes of 2µl in each well of both plates. Once the treatments were made, spike 1 of the inhibitors occurred, followed by an incubation for 30 minutes. Then, 200µl of the media was removed from the wells and the formulations pipetted gently on top, and lastly the spike 2 of the treatments occurred and the plates were places in the incubator overnight. After 24 hours, the reagents Luciferase and PrestoBlue were added to the wells and incubated before the plate reads on the TECAN.

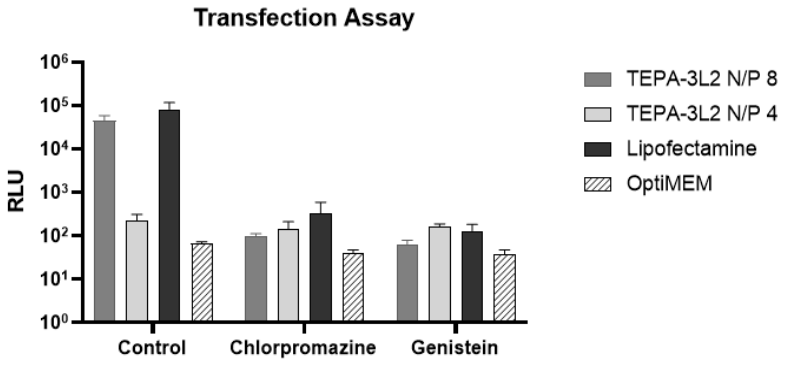

Figure 15. Transfection assays of TEPA-3L2 N/P 8 and 4, and controls Lipofectamine and Opti-MEM. Conditions consist of controls and inhibitors chlorpromazine and genistein.

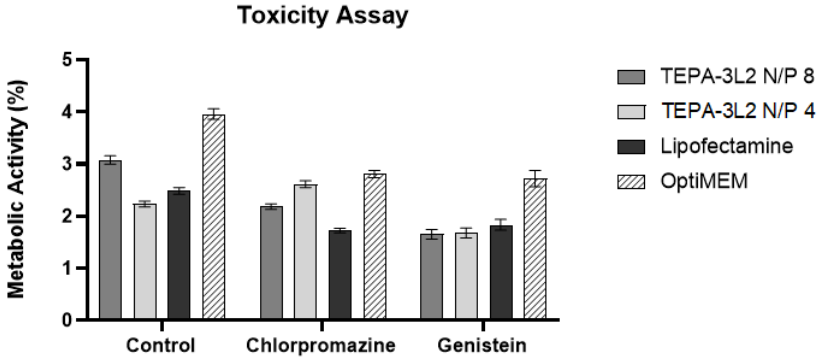

Figure 16. Cytotoxicity assay of TEPA-3L2 N/P 8 and 4, and controls Lipofectamine and Opti-MEM. Conditions consist of controls and inhibitors chlorpromazine and genistein.

The transfection assay displayed that both inhibitors reduced transfection efficiency of N/P 8 and Lipofectamine, whilst N/P 4 and opti-MEM exhibited a slight reduction in transfection (*Figure 15*). However, the results for N/P 8 correlated with the cytotoxicity assay (*Figure 16*), where addition of the inhibitors reduced metabolic activity, meaning the inhibitors might be toxic to the cells. These results indicate that the inhibitors may have potentially reduced transfection due to cytotoxic effects. The correlation of inhibiting the clathrin- and caveola-mediated pathways and reduced transfected indicates that the pathways may play a crucial role in the uptake of the PNPs, however cytotoxic effects need to be eliminated to establish their role. Further investigations into finding the concentration limit at which the inhibitor is still active, allowing for sufficient inhibition of endocytosis pathways with acceptable toxicity, can provide clarity.

3.8. CD7 images of chlorpromazine

Following the results from the transfection and toxicity experiments with the inhibitors (*Figure 15*), further investigation was conducted into chlorpromazine's effect on transfection in TEPA-3L2. Using a fellow academic's research into the polymer, genistein was excluded from further experiments due to the high cytotoxic effects accompanied with reduced concentrations. Furthermore, based on their results, N/P 16 and N/P 8 were used in this investigation as more endosomal puncta of GFP and CY5 was observed with the combination of chlorpromazine and the higher N/P ratio in comparison to N/P 4.

mCherry-GAL9 cells were seeded on a microclear plate, marked with the conditions TEPA-3L2 N/P 16 and 8, and controls of Opti-MEM, Lipofectamine, and free mRNA. All conditions had a plus chlorpromazine set of duplicate repeats and an untreated duplicate set. Using results from *Figure 15*, the concentration of chlorpromazine was further decreased to observe if cytotoxic effects could be reduced and sufficient inhibition of transfection would occur. A 1:7 ratio of inhibitor to PBS was used to make up 8µl, allowing for two spikes of 0.4µl to each of the wells. The same method was followed from previous inhibitor experiments, however after spike 2 of the inhibitor, 1µl of Hoechst was added to each well. The plate was then left to image overnight at 2 hour intervals in a temperature regulated ZEISS Celldiscoverer 7 confocal machine (CD7), and images for the channels GFP, mCherry, Hoechst, and CY5 were produced 24 hours later.

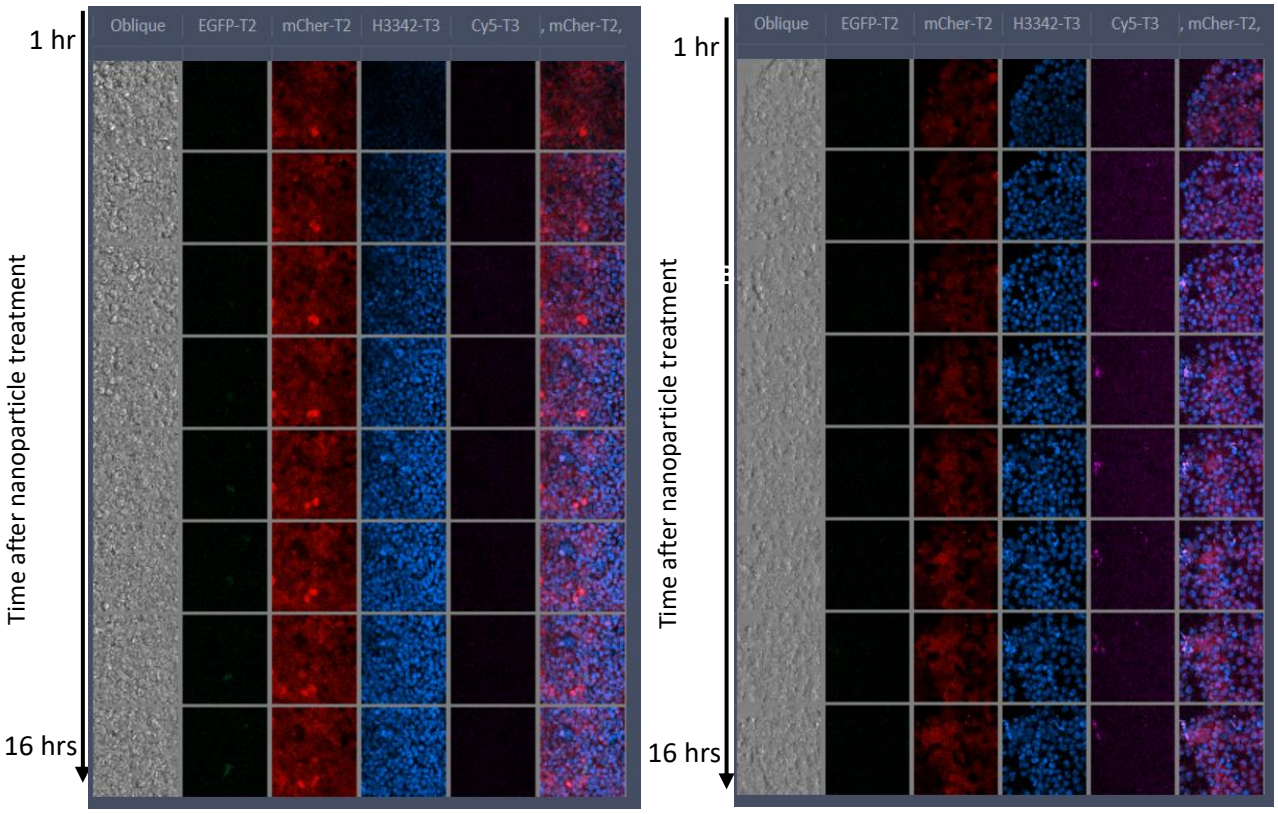

Figure 17. CD7 images of TEPA-3L2 N/P 8 with chlorpromazine (A) and without (B) with 2 hour imaging interval points. Imaging channels m-Cherry, Hoechst, Cy5 and eGFP can be observed.

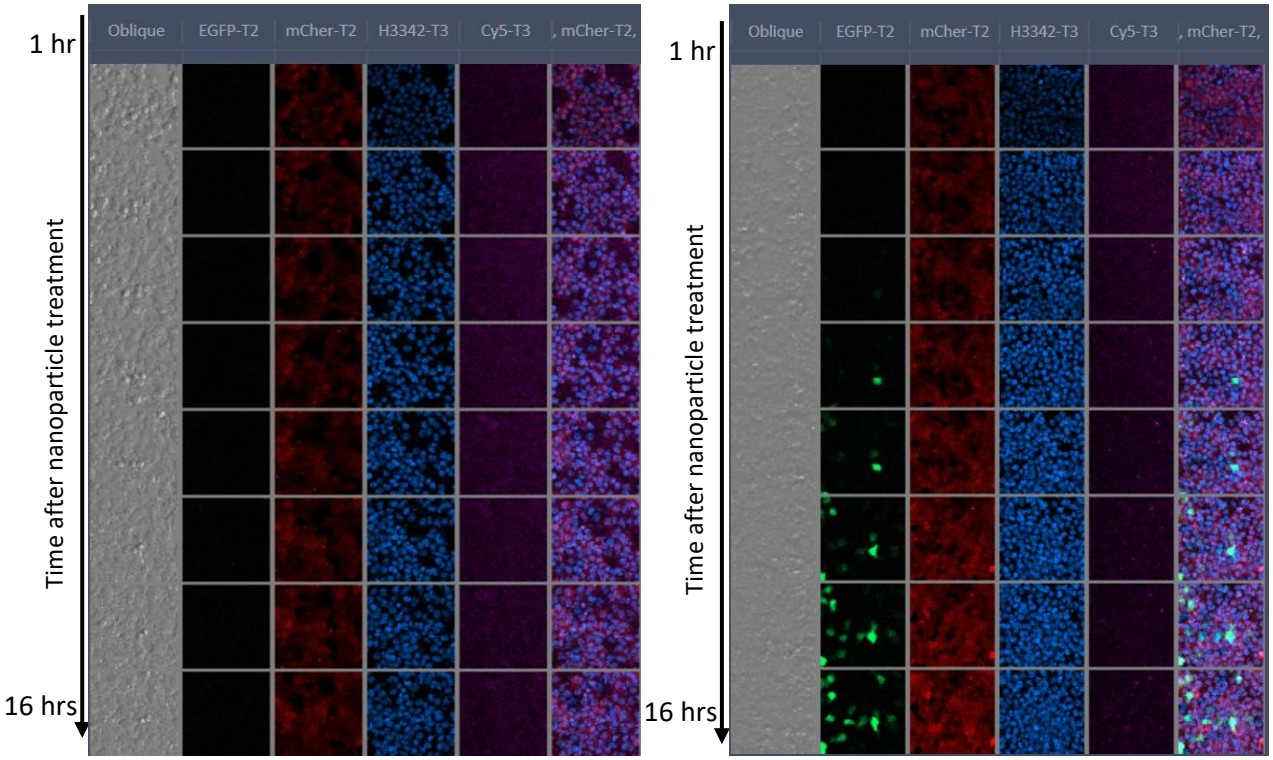

Figure 18. CD7 images of TEPA-3L2 N/P 16 with chlorpromazine (A) and without (B) with 2 hour imaging interval points. Imaging channels m-Cherry, Hoechst, Cy5 and eGFP can be observed.

The time-lapse microscopy captured images at 2 hour intervals to track the fluorescence intensity produced by CY5 and GFP and to observe the timescale of transfection. *Figure 17* displays images of TEPA-3L2 N/P 8 with (A) and without (B) chlorpromazine, whilst *Figure 18* exhibits N/P 16 with (A) and without (B) the inhibitor. Cy5 visualisation did not occur for the chlorpromazine condition of N/P 8 (*Figure 17A*), with slightly higher transfection visible in the control condition (*Figure 17B*). GFP expression was not visible in either condition, which was unexpected for the control as TEPA-3L2 N/P 8 has previously shown to successfully express GFP (*Figure 9*). The absence of GFP expression may have been due to experimental error whilst imaging on the CD7.

N/P 16 displayed a low amount of Cy5-mRNA visualisation and clear GFP expression that was visible at 12 hours in the control condition (*Figure 18*B). On the other hand, the treatment of chlorpromazine with N/P 16 indicated that GFP expression had been inhibited (*Figure 18A*). It is reasonable to assume that transfection was inhibited using a lower concentration of chlorpromazine in TEPA-3L2 N/P 16. However, due to no GFP expression being visible in the N/P 8 control, an experimental error must have been made, and hence the results cannot be evaluated against the treated condition. The conclusion whether inhibiting the clathrin-dependent endocytosis pathway being the bottleneck of transfection efficiency of TEPA-3L2 requires further investigations and a closer look at uptake mechanisms, which is explored in a later experiment.

3.9.1 RFP Concentrations

To obtain further knowledge about the structure of endosomal compartments and to observe endosomal puncta, the CellLight™ Early Endosomes-RFP tracker (BacMam 2.0, Invitrogen) was used. HEK293T cells were seeded on a microclear plate at the optimum density for an imaging experiment. After 24 hours, three conditions were used; 5µl of tracker per well in accordance with data from Invitrogen, 7.5µl and 10µl, to observe which concentration was ideal for treatment, at what time did uptake occur, and if clear endosomal puncta were observable.

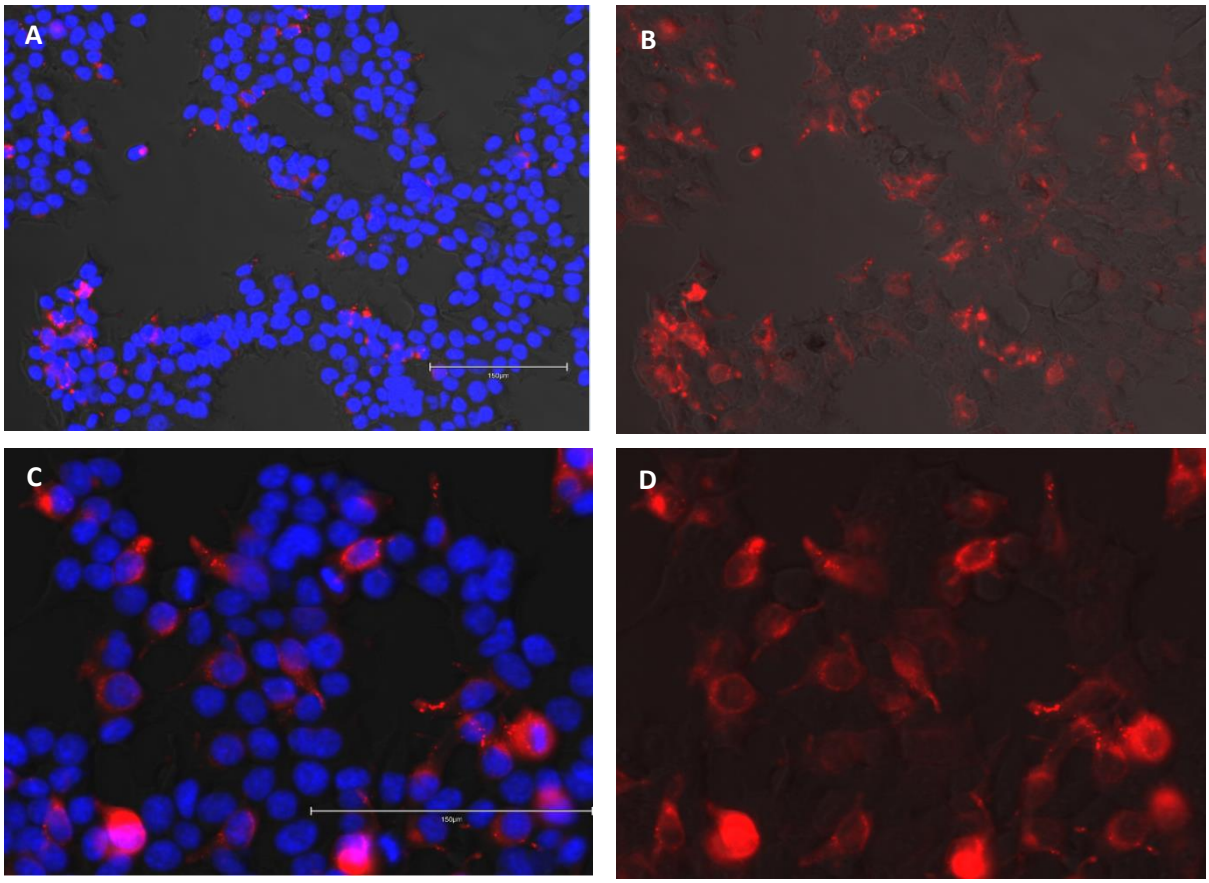

Figure 19. The ideal concentration of RFP tracker was found at 5µl per well. A and B display the cells at 20x magnification and C and D exhibit a 40x magnification. A and C are images of merged RFP, Brightfield and DAPI channels. Scale bar = 150 µm

The red fluorescent protein tracker allows for the visualisation of endosomal compartments, and endosomal puncta refers to dot-like structures observed within cells that represent endosomes. The RFP tracker showed uptake at 5µl concentration in the cells after 24 hours, with clear puncta visible in the early endosomes at 40x magnification, as seen in *Figure 19D*.

3.9.2 RFP tracker with chlorpromazine

Following the results of the RFP concentration study (*Figure 19*) and the reduced inhibitor concentration CD7 investigation (*Figure 15 and 16*), a smaller scale experiment using the RFP tracker and chlorpromazine was conducted to explore whether inhibition of transfection could occur, with the RFP probe allowing for visualisation in early endosomal compartments.

A microclear plate was seeded with HEK293T cells and treated 24 hours later with 4 conditions of TEPA-3L2 N/P 16 and 8, in accordance with the CD7 results, alongside controls Lipofectamine and Opti-MEM. Each condition had a control and 2 treatments; 0.8µl per well of chlorpromazine at the ideal concentration of 1:7 inhibitor to PBS, and a condition of 0.8µl of chlorpromazine plus the ideal concentration of 5µl RFP tracker. The wells underwent the spiking and treatment process, and 1µl per well of 1mg/mL Hoechst 33342 was added to stain nuclei followed by imaging on the EVOS M5000 at 6, 24 and 48 hours.

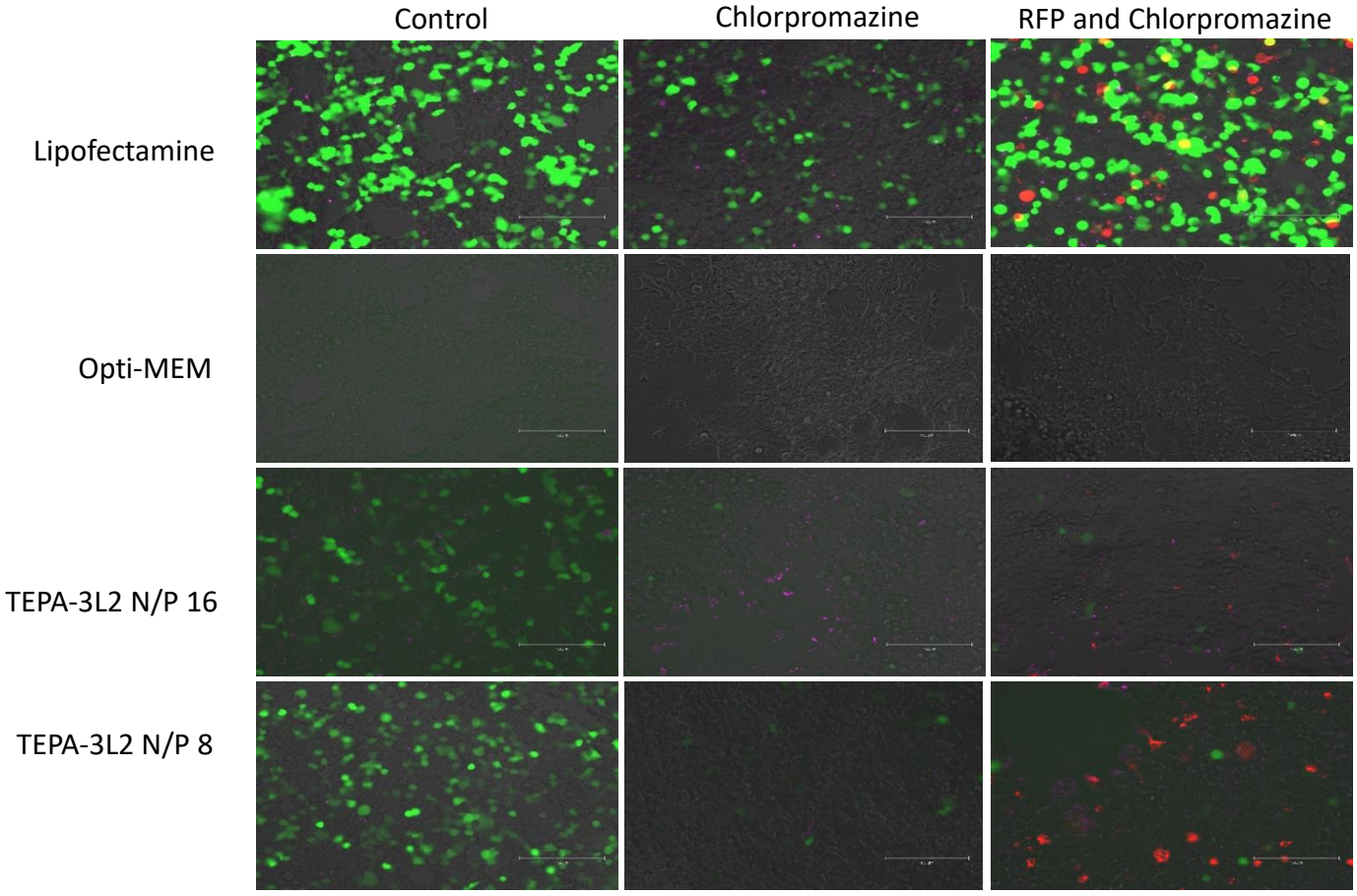

Figure 20. Images display the conditions TEPA-3L2 N/P 16 and N/P 8, alongside controls Lipofectamine and Opti-MEM. Treatments consisted of a control condition, treatment with chlorpromazine, and treatment with chlorpromazine and an RFP tracker. Images were captured 24 hours after treatment. Scale bar = 150µm

Imaging of the conditions was ideal at 24 hours, when clear GFP expression could be observed in the control conditions of lipofectamine, TEPA-3L2 N/P 16 and N/P 8 (*Figure 20*). Co-localisation between the RFP tracker, GFP and Cy-5 was not observed in either N/P ratio in the inhibitor plus tracker condition. The RFP tracker was visible with endosomal puncta particularly in N/P 8, but GFP expression did not occur in combination with the inhibitor. The lipofectamine conditions displayed that with the addition of chlorpromazine, there is less GFP expression and Cy-5 visualisation, whilst unexpectedly the lipofectamine, chlorpromazine and RFP condition displayed enhanced GFP expression. The reason for higher expression of GFP when combined with the tracker and chlorpromazine remains unclear.

Using a protein tracker and an inhibitor can potentially be toxic to cells, as RFP expression involves the synthesis and expression of a foreign protein, which can impose a metabolic burden on the cells that are simultaneously being affected by cytotoxic effects of the chlorpromazine. This may be a possible explanation of the reduction of GFP expression and Cy5 visualization in TEPA-3L2 N/P 16 and 8 when combined with the tracker and inhibitor. However it is unlikely, as decreased transfection was also exhibited in the CD7 images when chlorpromazine was used. Additionally, reduced cell viability was not visible in the treated conditions in *Figure 19,* as cells did not become more circular, indicating low cytotoxicity effects.

Overall, the combination of chlorpromazine and the RFP tracker in TEPA-3L2 N/P 16 and 8 displayed decreased transfection in comparison to the control, and no co-localisation of the nanoparticles with the early endosome tracker. These results coincide with the CD7 images where transfection was reduced in inhibitor treated conditions, suggesting that clathrin-dependent endocytosis may play a crucial role in uptake of the TEPA-3L2.

4. Concluding remarks

In conclusion, the experimental findings shed light on the current bottlenecks of polymeric nanoparticles efficiently delivering exogenous RNA into cells. Through an exploration of transfection efficiency and endocytosis pathways, valuable insights into how different N/P ratios of TEPA-3L2 and TETA-3L2 internalise in cells have been gained. The primary concept of the PNPs ability to deliver nucleic acid cargo was achieved for N/P 16, 8 and 4 for both polymers with no cytotoxic effects, establishing them as efficient nanoparticle vectors that can traverse the cell membrane and internalise mRNA.

The ability of the mRNA being transfected into the cytoplasm remains an issue. The potential of the endosomolytic agent chloroquine was explored to observe if transfection efficiency could be improved, as it is known to acidify the endosome resulting in subsequent endosomal escape of the nucleic cargo. The experimental findings display that transfection decreased for all N/P ratios of both polymers, potentially due to the accompanied cytotoxic effects of chloroquine. Further investigations into chloroquine through confocal imaging displayed that co-localisation of Cy5- and GFP-mRNA did not occur. Due to chloroquine not increasing transfection, endosomal escape is not the bottleneck of transfection using TEPA-3L2 and TETA-3L2, and further investigations into the rate limiting factor such as uptake or translation should be conducted.

Furthermore, investigations into endocytosis pathway inhibition were conducted to observe what pathway TEPA-3L2 uses to internalise in the cells. The insight of knowing which pathway is utilised allows delivery systems to be optimised to enhance uptake efficiency. The inhibitors genistein and chlorpromazine were investigated into inhibiting the function of the caveolae- and clathrin-dependent pathways, respectively. The inhibitors were tested with reduced concentrations as, whilst the transfection decreased, cytotoxic effects were present. Genistein's low transfection data could be interpreted as inhibition of caveolae-dependent endocytosis, but due to toxicity effects we can't be certain. Many studies that have previously used genistein fail to publish cytotoxicity results, so further investigations into optimising the inhibitor concentration in TEPA-3L2 may reduce cytotoxic effects and establish if caveolae-mediate endocytosis plays a role in uptake.

Alternatively, the reduced concentration of chlorpromazine displayed decreased transfection which was initially thought to be due to cytotoxic effects, however through RFP tracker imaging, it is visible that cells have not lysed. Therefore, decreased transfection is likely attributed to the inhibition of the clathrin-dependent endocytosis pathway impeding the internalisation of TEPA-3L2.

5. References

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