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BACHELOR'S THESIS

Biological Chemistry Bachelor's Program

mRNA expression of EGFP using Lipid nanoparticles

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Abstract

Two scientists, Katalin Karikó and Drew Weismann of Pennsylvania state university began research on mRNA as protein replacement therapeutics in the 90's, but due to the mRNA's immunogenicity it could not be used. However, in 2005 they found that the nucleotide uridine could be replaced with pseudouridine rendering the mRNA non immunogenic. When the pandemic hit, their work proved invaluable as many companies rushing to make the vaccines decided to use their technology. Their discoveries led to the first approved mRNA vaccines during the covid pandemic, saving many lives and earning them the Nobel prize in Physiology or Medicine 2023. Following this, RNA based therapeutics funding is now bigger than ever and researchers are hard at work seeking what other therapies RNA can be used for.

The technique used in the Sars cov 2 vaccine is cellular delivery by Lipid Nanoparticles (LNPs), a liposome like structure which can carry and deliver nucleic acids which are difficult to deliver by themselves. Among other factors the LNPs increase efficiency of delivery by increasing transfection, neither are they toxic to the body.

In this thesis we wanted to try to make these LNPs by ourselves and to see if we can express them in our chosen Human Colon Carcinoma cell line (HCT116). This was tested by seeding our cells with stained LNPs containing mRNA's that code for Enhanced Green Fluorescent Protein (EGFP) and imaging them confocally.

Expression of EGFP was achieved testing for which concentration was optimal, and in the subsequent run of the experiment we tried out different methods of LNP preparation. The results of the experiments showed that expression is possible and can be quite high. They showed that N1-Methylpseudouridine mRNA gave better expression in vitro than did 5-Methoxyuridine and that acetate buffer during LNP preparation seems to increase mRNA expression compared to citrate buffer.

Contents

Introductory:

Nanoparticles (NPs)

Nanoparticle is the generic term for particles of size up to 100 nanometres. In medicine the term nanotechnology is used to indicate nanoparticles made for therapeutic purposes. They are possibly multifunctional vessels that can be used in diagnosing and combatting cancer and other diseases[1]. They are also used as “Contrast agents” in imaging, for site specific imaging and to detect cancers. There are many medical practices that can only be performed via the use of nanoparticles, however they also bring their own risks and challenges[2].

Nanoparticles are used for several different purposes in scientific research, there are both established and new technologies for drug delivery and imaging using nanoparticles. There are many nanoparticle-based cancer medicines in clinical use. The nanoparticle formulation of paclitaxel (PTX) called “Abraxane” shows plenty of advantages over PTX and is approved for use in patients with metastatic breast cancer and non-small-cell lung carcinoma[3]. Interestingly they are also used for prolonging the effect of eye drops used post ophthalmic surgery or for treating Cytomegalovirus Retinitis. These nanoparticles showed increased drug retention time, improved efficacy, lowered toxicity, and experiments indicated they could deliver drugs to the back of the eye[4]. Clearly nanoparticles provide unique solutions to medicinal issues, through their small size they can accomplish things that larger particles cannot.

The main categories of Nanoparticles are: Quantum dots, used for site specific imaging; iron oxide nanoparticles, used in MRIs as a contrast agent for cancer detection; polymer- and liposome-based nanoparticles for drug and gene delivery, notably used for cancer therapy.[2]

Quantum dots:

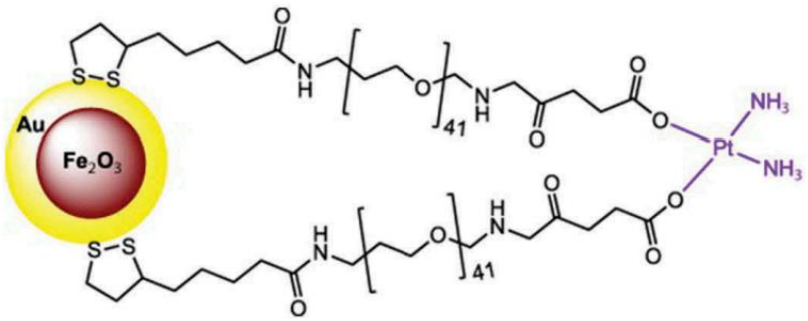
Organic dyes conventionally used in imaging are usually bound to biomolecules which locate and bind to certain cells or cell compartments, these fluorophores have issues with photobleaching and weak light intensity. Quantum dots are “inorganic semiconductor molecules” which possess stronger fluorescent light intensity. They are also resistant to photobleaching. Quantum dots are hydrophobic and are usually treated with a hydrophilic coating, which is then bound to affinity ligands that can take the construct to a select location within the body. Their discovery was celebrated with the Nobel prize in chemistry in 2023[2].

Iron oxide NPs:

Magnetic resonance imaging (MRI) scans do not require contrast agents in principle, but in practice it often is necessary due to similarities between neighbouring tissues in their magnetic properties. Iron oxide nanoaprticles (IONs) are contrast agents that can affect

select cells in a way that increases contrast in an MRI. IONs are also used as drug delivery systems, due to their high drug loading capacity and their properties including magnetism give them targeting abilities. Precisely produced IONs bound with therapeutic drugs exhibit efficient organ targeting and improved therapeutic effects (Vangijzegem T et al. 2019). Thereby overcoming the drugs' poor solubility and toxicity. IONs can be used to accumulate passively near leaky blood vessels caused by tumours but can also be localized to parts of the body by way of external magnets in active targeting.

As an example, in 2012 the group of Wagstaff and colleagues managed to synthesise iron oxide NPs to deliver cisplatin and achieved a 110x increase in cytotoxicity in human ovary cancer cells in vitro. First the Iron NPs were synthesised by coprecipitation and oxidized to obtain maghemite (Fe2O3), the particle was then gold coated. This was followed by a further particle coating of PEG lipids, the peg lipids serving as a linker providing strong bonding with the cisplatin drugs which were loaded onto the particle[5].



Picture showing a gold coated iron oxide NP fixed with cisplatin, an anti-cancer drug.

Drug and gene delivery by NPs:

Binding of therapeutics to nanosized polymer carriers has been widely tested since their first preparations in the 1970's. Compared to other NP types, these polymeric NPs (figure 1) possess greater architectural complexity and can thus be heavily tuned by modulating their components. They can perform "controlled drug release through diffusion or controlled degradation of the polymer matrix"[6]. There are many kinds of polymer NPs, and they show great promise as better ways to transport drugs, among other providing controlled delivery, higher loading capacity and higher efficiency after reducing drug dosage. In many techniques using preformed polymers, the first step is dissolving the polymer with organic solvents (figure 2). By removing this step, polymer NPs can achieve lowered toxicity by avoiding these compounds, thereby reducing an environmental risk[7, 8].

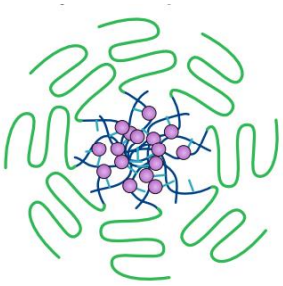


Figure 1. Example of a polymer np: here a polymeric micelle possessing a core-shell architecture. A hydrophobic core (violet) surrounded by a hydrophilic shell (green).[6]

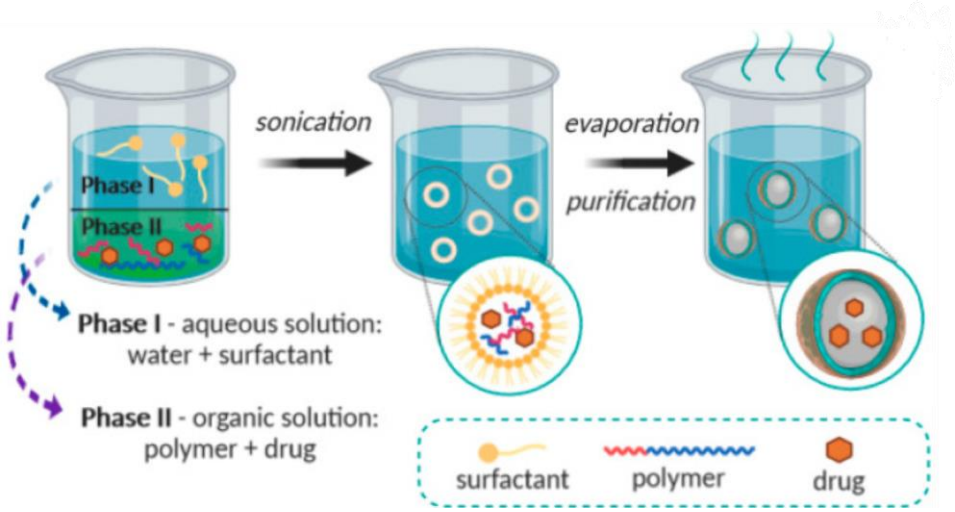


Figure 2. Example of Polymer NP synthesis by Solvent evaporation.

Liposomes

Liposomes are formed from a phospholipid bilayer encapsulating aqueous drugs in its core or lipophilic drugs in their membrane (Figure 3)[9]. This bilayer consists of phospholipids whose hydrophobic tails are in the middle and hydrophobic heads face out on both sides. There also needs to be cholesterol which sits in between the hydrophobic tails stabilizing the liposome and modulating membrane permeability.[10] They can also contain membrane proteins and polymers. This phospholipid bilayer is similar to the one that encapsulates the cells in our bodies, allowing liposomes to fuse with our cell membranes for delivery of the contained drugs or mRNA.

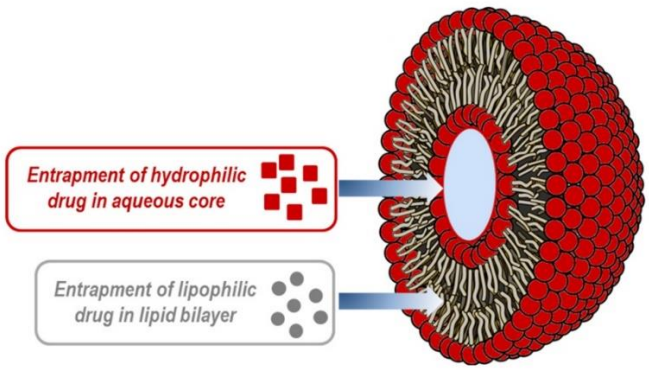


Figure 3. Liposome: The red heads of the phospholipids are hydrophilic and the grey tails are hydrophobic, this layering is formed naturally due to these properties.

Lipid Nanoparticles

Lipid nanoparticles (LNPs) are liposome-like particles utilized for delivery of nucleic acids. They differ from liposomes in that they form a micellar structure internally, the composition of which can be altered through different formulas and parameters when synthesizing[11]. LNPs mainly consist of 4 components: 1. Phospholipids for the general structure of the LNP. 2. Cholesterol for stability in vivo as well as helping intracellular delivery. 3. Ionizable or cationic lipids to bind the negatively charged genetic material in the core, and to aid in endosomal escape. 4. PEG (polyethylene glycol) lipids providing colloidal stability and increasing circulation time, however they may have a negative effect on uptake and endosomal escape[12].

LNP composition

Focusing on LNP uptake, toxicity and biodegradability, there are many choices that need to be made with the end goal of achieving high transfection and expression in cells. LNPs contain phospholipids to make up the bilayer membrane, and other lipids with specific qualities are mixed into and on that membrane.

The first part is ionizable lipids that consist of an ionizable head group, a linker region and a hydrophobic lipid tail (figure 4). A study by Moderna claims that an ionizable lipid pKa range between 6.2-6.6 is ideal for protein expression if administered intravenously[13]. It is hypothesized that the linker region can have an effect on head group pKA and on endosomal escape. As for the hydrophobic tails there have been implementations of ester linkages in their structure that can be cleaved in vivo by esterases, decreasing toxicity by making the LNPs more biodegradable[14].

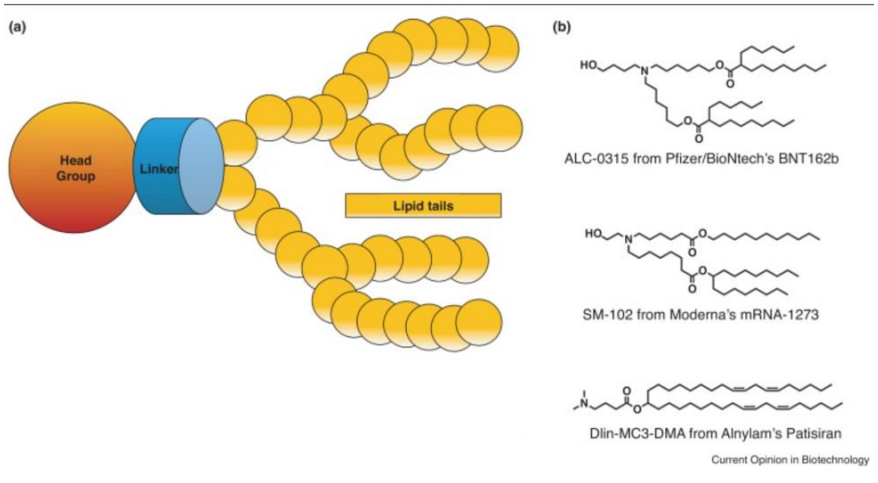


Figure 4. The general outline of an ionizable lipid, the bottom right structure is the ionizable lipid used in this experiment: Dlin-MC3-DMA.

Polyethylene Glycol (PEG) lipids (figure 5) are another type of lipid needed to make LNPs, they will affect the size of the LNP and the stability of it. Using different PEG lipids can lead to differences in these attributes, including longer or shorter circulation times and may even affect cellular uptake and endosomal escape[15].

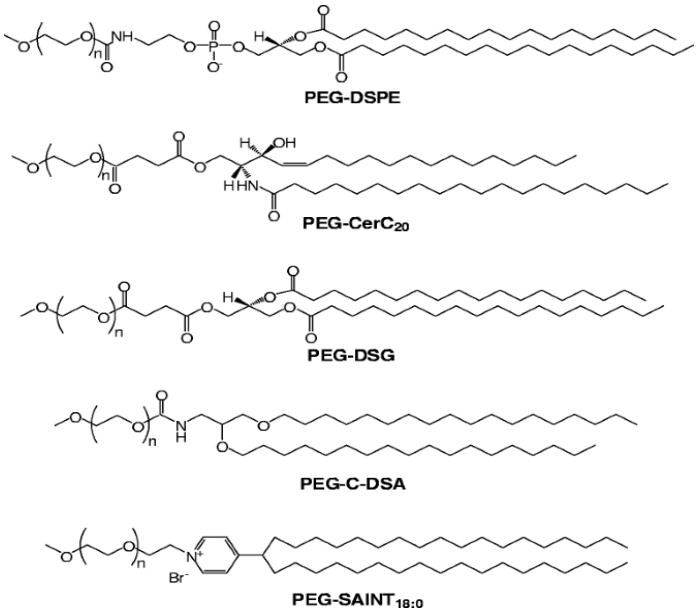


Figure 5. Examples of PEG lipids.

Cholesterol is another integral part of LNPs, and it can affect various properties of the nanoparticle. LNPs with cholesteryl oleate (figure 6) provide specific biodistribution of LNPs by providing higher selectivity for liver endothelial cells than for hepatocytes[16]. Oxidative modifications on the Cholesterol tails can also enable the LNPs to accumulate more in endothelial cells of the liver and in liver resident Kupffer cells[17].

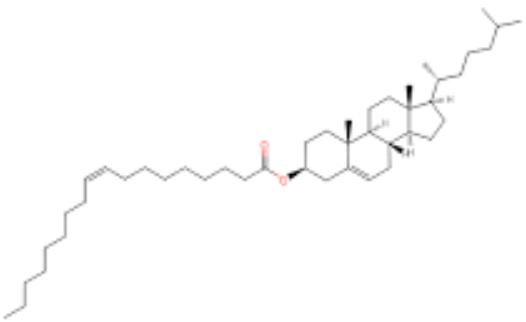


Figure 6. Cholesteryl Oleate

The LNPs are carrier particles, and they are made for carrying oligonucleotides like siRNAs, mRNAs or proteins (figure 7).

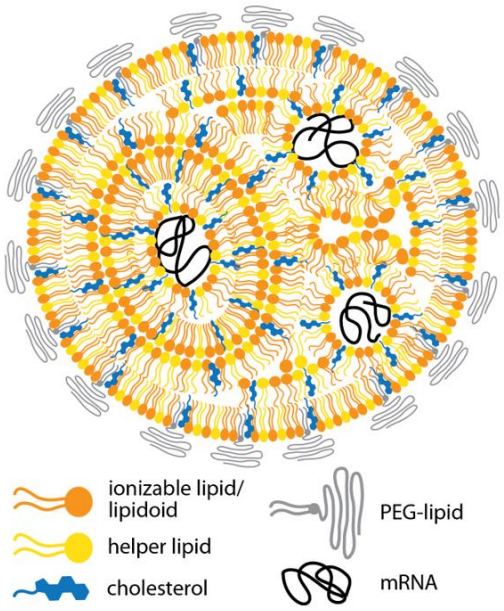


Figure 7. mRNA lipid nanoparticle with its different components.

mRNA

DNA is the genetic material which resides in the nucleus of our cells. It contains the recipe for who we are and for the functions our bodies carry out daily. In a human cell there are 46 long DNA strands called chromosomes. These chromosomes which are usually very condensed, can be unwound for access of RNA polymerase II the protein complex responsible for the transcription of mRNAs. These mRNAs are single stranded copies of DNA segments apart from the presence in them of the nucleotide uracil in place of thymine. The body makes these mRNAs which can pass through the nuclear envelope into the cytoplasm where the ribosomes reside. Ribosomes can translate nucleotide sequences on the mRNA strand into the corresponding polypeptide sequence or protein. The protein can then serve a purpose within the cell or outside it. So effectively the mRNA is the intermediary between DNA and protein expression. For many decades mRNA's have not only been made in vivo, but are also synthesised in vitro and their nucleotide sequences can be modified so they do not cause immunogenic reactions in the body, allowing further study of them and allowing their use in medicine.

mRNA as a drug

The idea of using nucleic acids as drugs was first conceived over 30 years ago, when Wolff et al managed to express proteins after injecting mice with in vitro transcribed mRNA and with plasmid DNA (Wolff, J.A., et al, 1990). This resulted in protein expression without even a dedicated delivery system[18]. However at the time the concept was not

further researched due to low stability of RNA compared to DNA, a clear disadvantage when used as therapeutics. We know now that in vitro transcribed mRNA carries multiple advantages over plasmid DNAs and other DNA based therapeutics. The mRNA does not need to enter the nucleus, once it has made it to the cytoplasm it can then be translated, while DNA needs to enter the nucleus. mRNA doesn't carry the risk of integrating into the genome causing insertional mutations, and does not have a permanent effect, on the contrary, mRNAs are transient, getting slowly degraded in the cytoplasm. Because of this they can be administered as needed, and mRNA manufacturing have become relatively cheap over the years.[19].

Opposed to Wolff's experiment on rats, the application of mRNA-based therapeutics to humans necessitates an mRNA complex that is non-toxic, has high transfection, is transient/biodegradable, is relatively cheap and easy to produce, and can be stored long term. For all this a carrier nanoparticle is needed.

mRNA carrying capacity of LNPs

To inject mRNA into the bloodstream and have it eventually translated into proteins requires the mRNA strands be transported safely to and through the cell membrane before being released into the cytosol. Just looking at the cell membrane only 1 in 10 000 of the initial mRNA molecules would make it through the membrane by diffusion due to its negative charge[19]. If the mRNA is encapsulated in an ionizable LNP, the amount of mRNA making it through becomes much more significant. Travelling through the bloodstream it will no longer interact with other particles and components in the plasma. When reaching the cell membrane which has a much higher affinity for the neutral LNP particle it is then easily transfected. After internaliziation it gradually reaches acidic endosomal compartments where the LNP becomes suddenly charged allowing for endosomal escape and release of mRNA into the cytosol[20]. In addition to the ionizable LNPs ability to get the genetic material into the cell, they are also very easy to synthesize, requiring mostly basic laboratory equipment. Although the ingredients are not very cheap to obtain

LNP entry into cells

From the moment the LNPs are synthesized the lipidic structure protects the internalized mRNA from degradation by RNases through electrostatic interactions keeping it stabilized in the core. Furthermore, integration of PEG lipids keeps the Mononuclear Phagocyte System (MPS) from recognizing it and helps it from being filtered off in the kidneys[21]. They can be conjugated to specific antibodies, so they localize to a particular cell type[22].

After reaching the targeted cells different types of internalization can commence. The general types of endocytosis are Phagocytosis both passive and receptor mediated, pinocytosis and receptor mediated endocytosis.

Phagocytosis is performed by some immune-cells and is a form of endocytosis of harmful bacteria, dead cells and debris, it is performed through an extension of "Pseudopodia", an extension of the cell membrane wrapping around and engulfing particles. This nanoparticle is thus engulfed into a vesicle which fuses with lysosomes to effectively eat the particle.[23]

Pinocytosis functions as nonspecific uptake of extracellular contents into the cell through an inwards pinching of the cell membrane taking with it small particles mainly for consumption via fusing the newly formed vesicle with a lysosome.[23]

Most cells have hundreds of different types of receptors in their cell membranes allowing for selective uptake of different contents. Once ligands are bound to their complementary receptor, these bound receptors cluster together and are eventually engulfed in an endosome. As the particles are released the receptors are then recycled to the cell membrane. This is useful for obtaining large concentrations of a certain particle that exists in low concentrations outside of the cell.[23]

Lipid Nanoparticles are within the size range that they would be engulfed into the cell through pinocytosis or receptor mediated endocytosis, but despite their small size they are thought to be taken up by phagocytosis (Griffiths et al, 2022).

Endosomal escape

The mechanism for release of mRNA into the cytoplasm is not well understood although there are two general theories. The first theory revolves around the ionizability of LNPs when in the acidic compartment of the endosome.

The pH in these endosomes is generally lower than physiological starting at around 6 in early endosomes, slightly lower for larger endosomes, and down in the 4 range for lysosomes, although this will vary between cell types. Now although the mechanism for the release of mRNA from the LNP and endosome is not yet fully understood, it has been suggested that the low pH of the endosomes combined with the ionizability of the LNPs plays a major role in it. When in the acidic environment of the endolysosomal system, the now cationic LNPs interact with the Anionic lipids of the inner endosomal membrane forming structures that allow for release of LNP contents[24, 25]. DLin-MC3-DMA, an ionizable cationic lipid was found to be the best suited lipid when tested with mRNA for protein expression[26]. The parts remaining in the endosome end up fusing with a lysosome and get degraded.

The other theory is called the proton sponge effect, which is based on the buffering ability of LNPs activating proton pumps in the endosome causing an influx of negative

chloride ions, which again increases the osmotic imbalance leading to water intake and eventual bursting of the endosome, releasing the mRNA containing LNP (figure 8)[27].

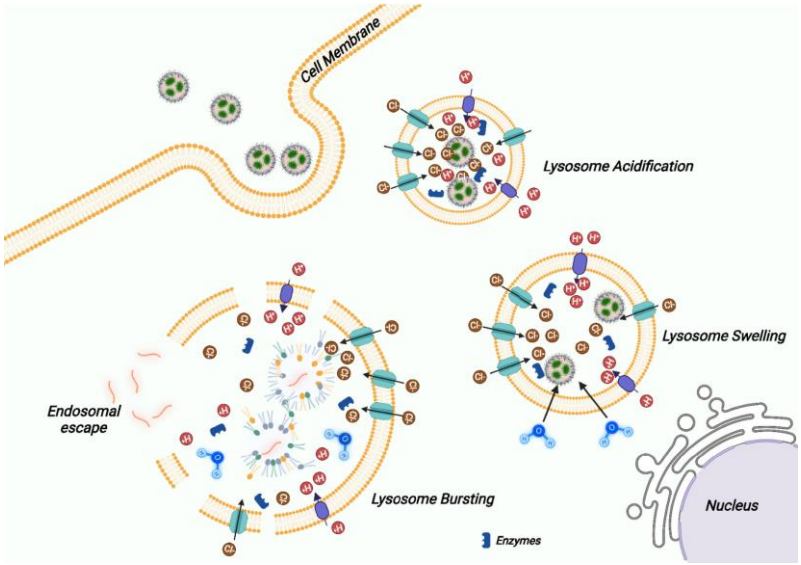


Figure 8. The proton sponge effect.

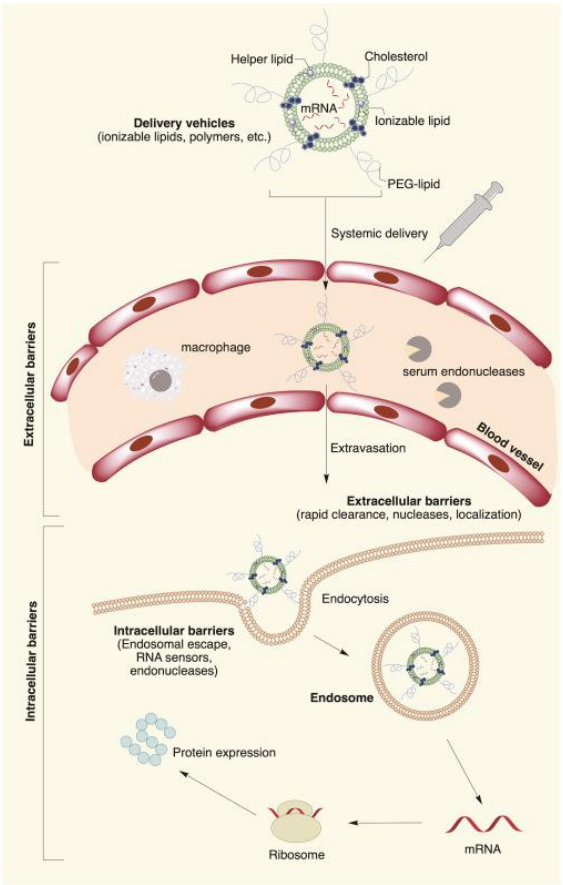


Figure 9. Illustration showcasing the journey of an mRNA containing LNP from systemic delivery into the bloodstream, leaking into tissue by extravasation and entering a cell through endocytosis, winding up in an endosome inside the cell. After escaping the endosome the mRNA is used for protein synthesis by the ribosome.

HCT116

The aim of this thesis is to test if we can get expression of EGFP in vitro via LNP containing EGFP mRNA and optimize the method of LNP preparation for obtaining the maximum expression. A good place to start is by choosing what cell types to work on. Low efficiency of mRNA expression in cells would limit our ability to optimize the process of LNP making. We have therefore chosen a human colon carcinoma cell line (HCT116) that has been shown in a paper from 2019 to have a very high efficiency of mRNA expression from mRNA carrying LNPs[28]. Their data showed that while HCT116 achieved transfection and expression in more than 50% of cells within 4 hours, another cell line tested (H358) achieved the same after 36 hours, and yet another (CT26.WT) only achieved 5% after 36h.

Confocal Imaging

The first functional confocal microscope was invented by Marvin Minsky in 1955, it was made to be able to image live tissue in real time. Confocal microscopy is a sub section of fluorescence microscopy. The benefits compared to conventional optical microscopy are a shallow depth of field, filtering off the out of focus glare and taking a photo series in the z-plane section by section. This enables the microscope to capture short slices of an object in high focus, without interfering glare. The microscope can gather a stack of pictures breaking down an entire sample into tiny photos that can be looked at individually or put together as one 3D image.

Confocal microscopes are said to bridge the gap between optical imaging and electron microscopy, providing higher resolution than the former but less resolution than the latter. Confocal microscopy is however more prone to photobleaching.

Light Scanning Confocal Microscopy is a highly technological tool, and the microscope needs to be accompanied by laser systems and other machines and is controlled by a computer (Figure 10). Powerful computer software enables utilization of the microscope and makes it as easy as possible, using specialized software one can practically control the entire process. The software enables the selection of laser wavelengths, laser intensity and gain, it can control the size of a z-stack and how small and how many slices the stack should be built of. The setup can take multiple stacks of the same frame with different laser wavelengths to excite and differentiate between stains and autofluorescence.

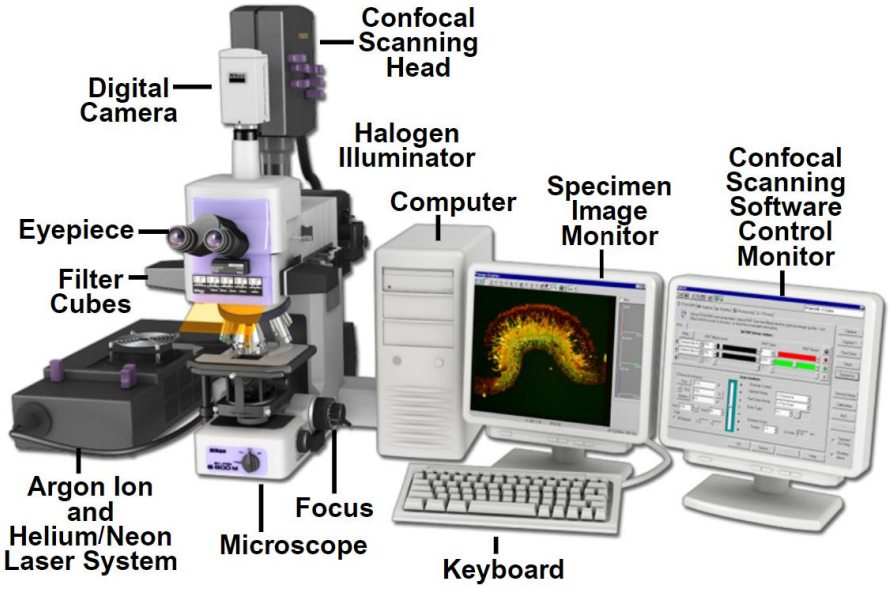


Figure 10. An example of a confocal microscope setup[29].

The microscope works by sending light from a laser through a collimator that narrows the beam of light, this light hits a dichromatic mirror reflecting the short wavelength beam to a scanning mirror (figure 11b) above the objective lens. This mirror and objective lens scan the laser over the sample to build the image. The emitted fluorescent light from the sample is passed back through the objective lens and now through the dichromatic mirror due to the emitted light having a longer wavelength, through a pinhole to eliminate out of focus light and finally reaches the Photomultiplier detector (figure 11a)[30].

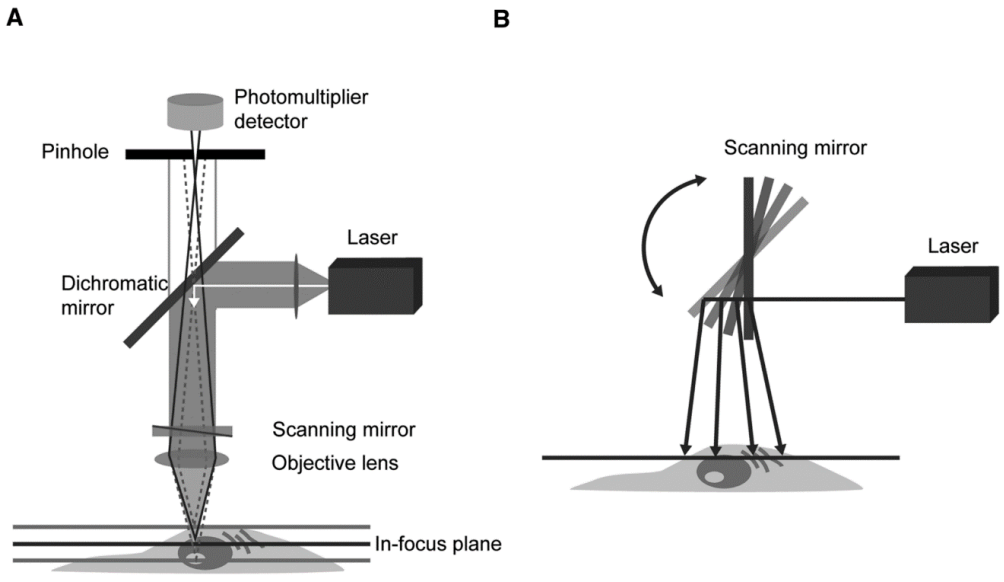


Figure 11. (a) Photo showing the general mechanism of a confocal microscope. (b) Showcases the mirror scanning the laser over the sample area[30].

After the emitted light reaches the detector, the signals are passed back to the computer and compiled into a photo. Imaging analysis software can then be used to analyse captured photos and stacks. Fiji is a program using the core of ImageJ with additional plugins that is commonly used because it can manipulate confocal images for quantification and other analyses. It's relatively user friendly and has a dedicated user base.

Fluorescence

When the confocal microscope was invented, fluorescent dyes were long since discovered and already used for microscopy. Fluorescence can be described as the light obtained from a molecule which absorbs light and almost simultaneously re-emits it due to a short time span between photon absorption and emission.

Fluorescence was not something created in the laboratory, many objects display what is called autofluorescence, the word used for fluorescence found naturally, in minerals, crystals, animals and our own bodies. It occurs due to proteins and other natural structures which can absorb and emit light. This autofluorescence becomes an issue when doing fluorescence microscopy due to the generated fluorescent noise, the fluorescence from an emitting a dye will always be detected among some background light. To minimize this, a multitude of fluorophores exist so you can choose ones that result in less noise (figure 12). They are commonly used dyes which only excite from light at a certain wavelength, minimizing the noise through only exciting the sample with one wavelength. There are many fluorophores for different wavelengths and with different properties, so finding the right one for your experiment may be difficult, especially if the experiment requires more dyes.

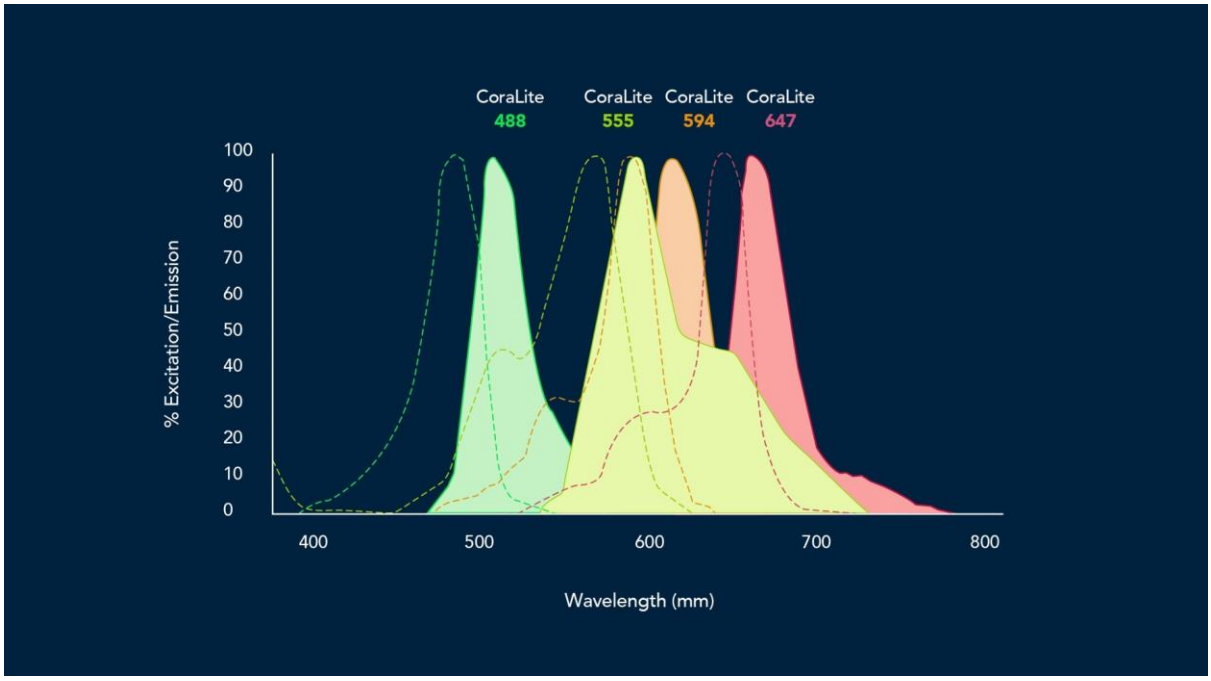


Figure 12. Clear overlap between some of the dyes in this image. Stapled line is excitation and filled wave is emission.

The emission light of a fluorophore usually has a higher wavelength than the excitation source, this is due to vibrational energy loss when photons fall back to ground state. This difference in wavelength is called Stokes' Law or Stokes' shift. The higher the Stokes' shift is, the easier it becomes to split emission from excitation light with fluorescence filters. However, this difference in emission light from the excitation source is problematic when multiple fluorophores are involved in an experiment. Both the excitation and emission is a spectrum, and dyes will also absorb and emit light at other wavelengths near the ones they are meant to. This means that two different dyes could absorb and emit light at the same wavelength between their respective peaks. This will lead to what is called crossover in our results, the images will display bleeding through between channels and it will become harder to draw conclusions from them[31].

When choosing dyes it is thus important to choose ones that minimize crossover. This goes also for choosing dyes whose spectra do not cross over with the spectra of auto-fluorophores. In biotechnology, scientists often choose to express the variants of the protein GFP (Green Fluorescent Protein), the gene for which was cloned and expressed from the Aequorea Victoria or crystal jellyfish. It absorbs light at 395nm and 475nm and emits green light, peaking at 509nm. When working with these proteins the scientist must again avoid crossover when choosing a dye.

This organic dye has since its discovery been greatly modified through protein engineering. Among the desired improvements were faster folding at physiological temperatures and brighter shine, which is accomplished in EGFP, E standing for enhanced. EGFP also has a different excitation spectrum than regular GFP, absorbing light at 488nm while still emitting at 509nm (figure 13). EGFP is the protein used in this thesis. Emerald FP which has increased photostability and even brighter shine. And the "superfolder" FP which folds faster than, is brighter than and is more acid resistant than the former variants. These improvements are accomplished by mutations in different parts of the proteins' amino acid chain[32].

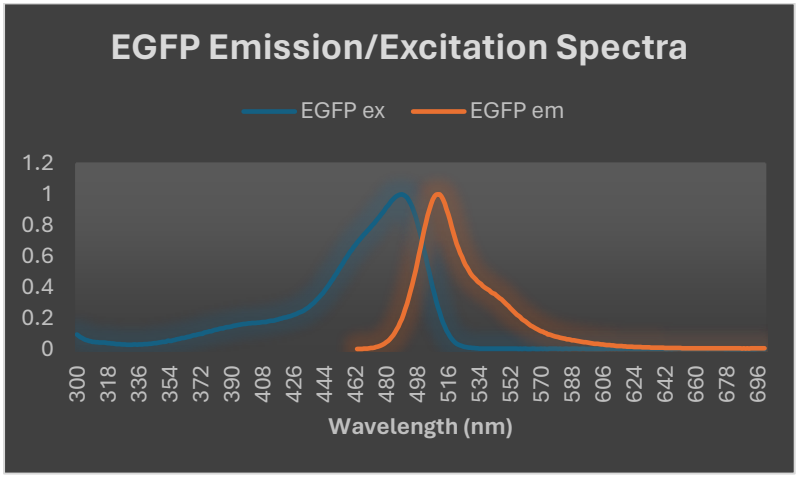


Figure 13. Emission and excitation spectrum of EGFP.

Goals

This thesis focuses on lipid nanoparticles for delivery of mRNA, this includes synthesising of different LNPs and figuring out what compositions provide the highest amount of expression achieved by the LNPs through testing them on cells in vitro.

Materials and Methods:

Cell culture

A culture of HCT116 cells was started in mid-January of 2024. Over the months this cell culture was kept alive through routinely changing media, counting, passaging and freezing cells if a new cell culture needed to be started. The cells were acquired at passage 14.

The culture was maintained using standard cell culture procedures, ensuring to avoid contamination. The media used was DMEM Complete with L-Glut and 10% FBS. When passaging PBS was used to clean off debris, and Trypsin was used to loosen cells from the flask. For counting I used a MUSE cell count and viability kit. We used culture flasks, Eppendorf tubes, larger 50ml vials for aliquots and smaller 10-15 ml vials for different procedures. Imaging dishes were used for imaging with an SP8 Falcon Confocal Microscope utilizing a 20x dry lens.

Making LNPs

The making of our Lipid Nano Particles consisted of thoroughly mixing together a water phase containing the mRNAs in an acetate buffer or citrate buffer, with an ethanol phase containing all the lipids in pure ethanol. We used two different types of EGFP mRNA, one modified with 5-Methoxyuridine (5-mRNA) and one with N1-Methylposeudourtidine (N1-mRNA). An overview of the water and ethanol phases can be found in the table below.

Ethanol phase	Mol%	Concentration mg/ml	Volume added (µl)
D-Lin-MC3	50	25	15.8
DSPC	9.9	20	4.81
Cholesterol	38.5	20	9.2
DOPE-ATTO-550	0.1	1	1.6
DMG-PEG	1.5	20	2.3
Ethanol			33

Total volume 66.7µl

Water phase	Weight to encapsulate	Conc.	Volume added (µl)

mRNA	0.02mg	1.7111g/mol	11.7
Acetate/Citrate buffer			121.6

Table 1. Total volume 133.3µl

First dissolve all lipids in the ethanol phase, then dissolve the mRNA in acetate buffer for the water phase. When ready, pipette the ethanolic phase into the mRNA solution while vortexing the solution, and LNPs will form instantly. These LNPs are prepared with a 10:1 concentration of ionizable amine in the ionizable lipid to anionic phosphates in mRNA. To get rid of the ethanol the solution was dialyzed overnight in de-ionized water using a Pur-A-Lyzer™ dialysis kit (MW 6000).

Acetate buffer preparation. A 100ml pH 4.0 Acetate buffer was made by dissolving 20mg of sodium acetate in 80ml of DNase and RNase free water. Mixing in 66.7ul of acetic acid and adjusting the pH to 4.0. Finally topping up to 0.1 litres with more water.

Citrate buffer preparation. A 100ml pH 4.0 Citrate buffer was made by preparing 80mL of DNase and RNase free water. Mixing in 993 mg Sodium citrate dihydrate and 1.273 g of citric acid and adjusting to pH 4 with HCL and NaOH. Finally topping up with more water until the volume is 0.1 litres.

To summarize. two lab experiments were conducted on the cells. Other than the differences in table 2 below, the experiments were conducted in the same way.

Concentration test	Buffer and mRNA test
4 different LNP concentrations: 1:8000 1:4000 1:2000, 1:1000	Only one concentration: 1:1000
Only one mRNA: 5-Methoxyuridine in acetate buffer.	Four different mRNAs: 5-Methoxyuridine in citrate buffer 5-Methoxyuridine in acetate buffer N1-methylpseudouridine in citrate buffer N1-methylpseudouridine in acetate buffer
ATTO-550 fluorescent dye was used	No dye

Table 2

Before the second experiment, a new cell culture was started with the frozen cells. It was done simply by retrieving one vial of cells from the liquid nitrogen tank. Heating it slightly by swirling in the water bath until the ice was just melted. Then the supernatant was mixed with new media and put in a cell culture flask.

Seeding cells with LNPs

For the first run of the experiment, we seeded 5 imaging dishes with cells while passaging. And after about 24 hours of incubation later we came back to remove the old media and to apply new media mixed with LNPs, keeping one control sample and seeding the others at 1:1000, 1:2000, 1:4000 and 1:8000 Dilutions (LNP-volume: total volume) before leaving them to incubate for another 24 hours.

After analyzing what concentrations gave the best expression the experiment was repeated with new batches of LNPs. Now all at 1:1000 volume concentrations, but with slightly different components (see table 2).

Imaging with confocal microscope and analyzing with Fiji

Twenty-four hours after LNP seeding the dishes were imaged with a Falcon SP8 confocal microscope. four z-stacks with the same parameters were taken of all the dishes. All images were taken with the same program settings, however in the last experiment the acetate buffer samples proved to emit fluorescence that maxed out at 255 intensity. These images were taken twice, with a reduced signal amplification gain from 125% down to 10% so we could observe the difference between N1-mRNA and 5-mRNA in acetate. These images were then analysed in an image analysis program called Fiji and the fluorescence was quantified. The analysis in Fiji was done by compiling all the images in a stack into one image using the maximum intensity of each pixel. Taking four set size frames of Regions of Interest (ROI) of all images and measuring the amount of light emitted in that frame, then calculating the average of them all and subtracting from that the average fluorescence in the areas without cells, effectively removing the background noise.

Results

In the first set of experiments we saw clear expression of EGFP, but we were also surprised to see that the lowest concentration (1:8000) sample was giving good expression and the 1:2000 concentration sample had barely any. Doubtful to these results, the experiment was repeated the following week, giving clearer results that were more in line with the fact that higher concentration should lead to higher expression (see figures 14-18).

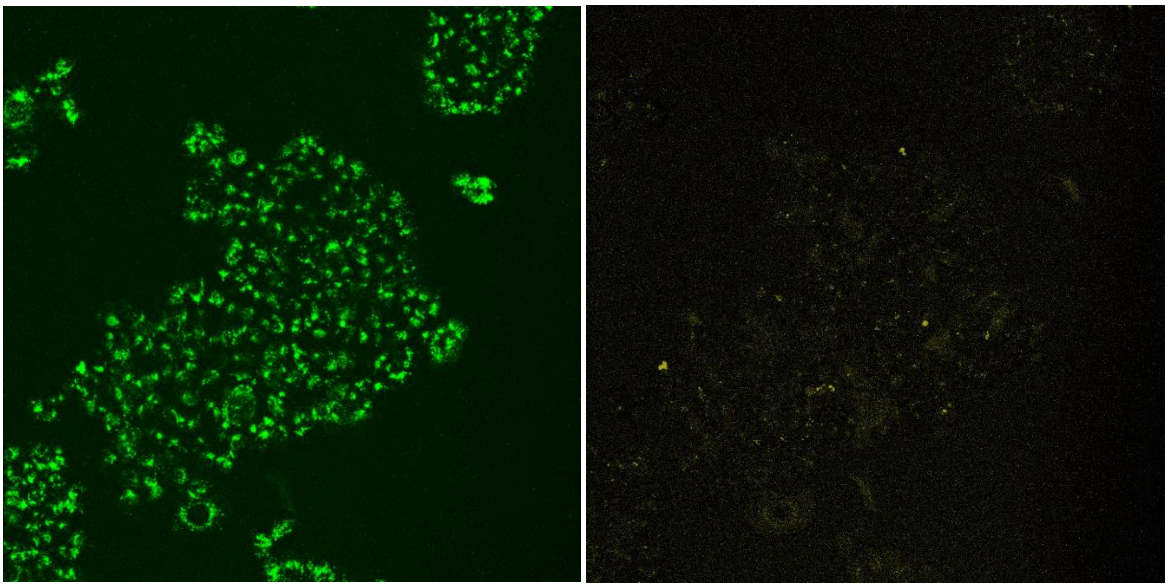


Figure 14. 1:8000 concentration, left picture showing labelled LNPs in green. Right picture in yellow showing maybe a little EGFP expression, but it is hard to discern if it is not just crosstalk from the LNPs. Image acquired with a 20x lens with SP8 confocal microscope.

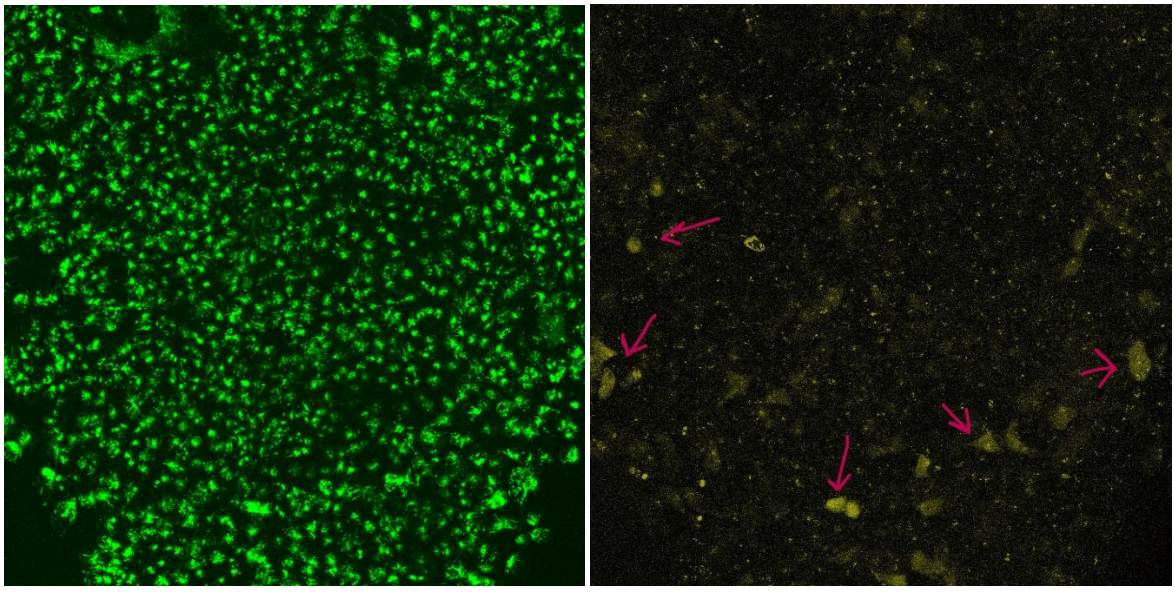


Figure 15. 1:4000 concentration, in the right picture in yellow we clearly see some cells where there is EGFP expression and not just crossover. Red arrows indicate some of the cells expressing EGFP.

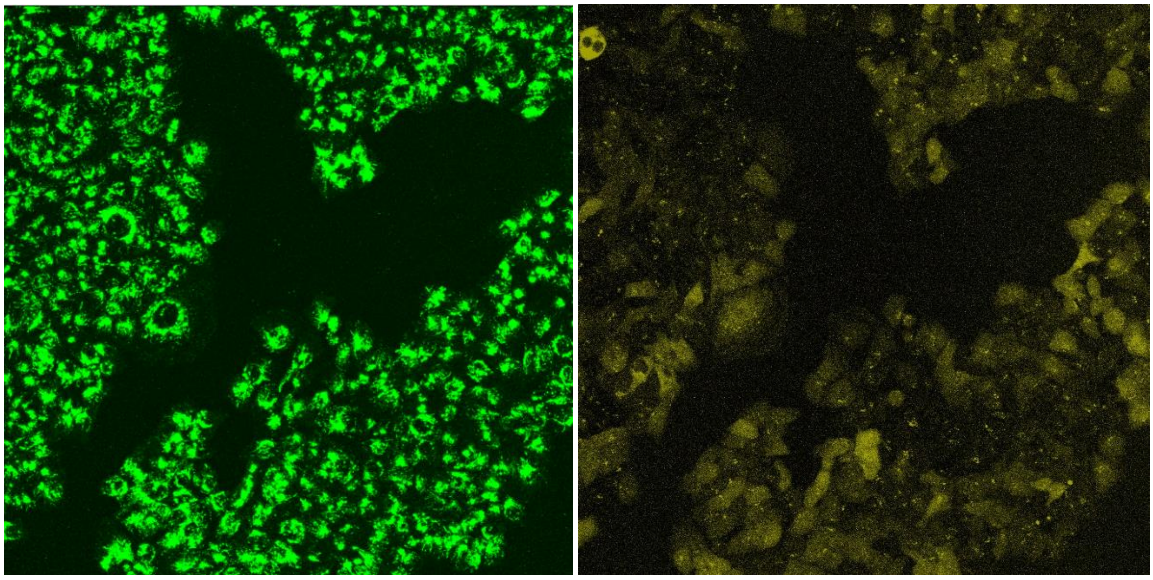


Figure 16. Green fluorescent LNPs to the left and Yellow EGFP to the right. 1:2000 concentration, here more than half of the cells show expression of EGFP.

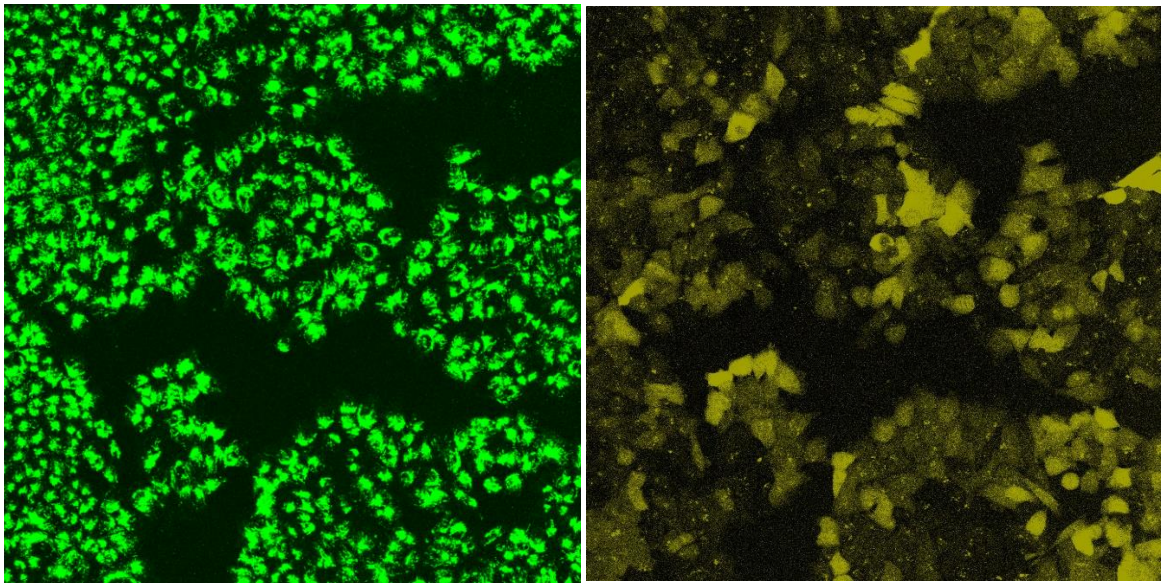


Figure 17. Green fluorescent LNPs to the left and Yellow EGFP to the right. 1:1000 concentration, expression is at its highest here.

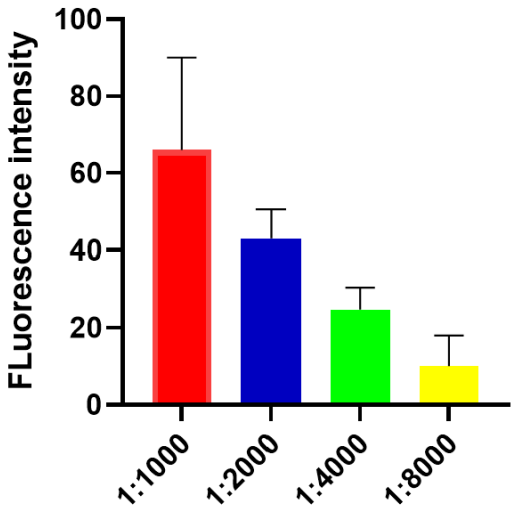


Figure 18. Comparison of fluorescent light emission between different concentrations. We could assume normal distribution and thus did a parametric t-test on the neighbouring concentrations (1:1000 to 1:2000, 1:2000 to 1:4000 and 1:4000 to 1:8000), (n=16).

Welch's t-test gave that $P < 0.05$ for 1:8000 to 1:4000 and for 1:4000 to 1:2000, although for 1:1000 to 1:2000 the t-test gave $P=0.15$. After deciding from the photos (figure 16 & 17), the graph (figure 18) and from the statistical data that 1:1000 concentrations gave the best expression, it was also the concentration used for the following experiment testing different types of mRNA and citrate buffer vs acetate buffer. Knowing we would get expression, the LNP dye was left out this time around to avoid crosstalk between channels.

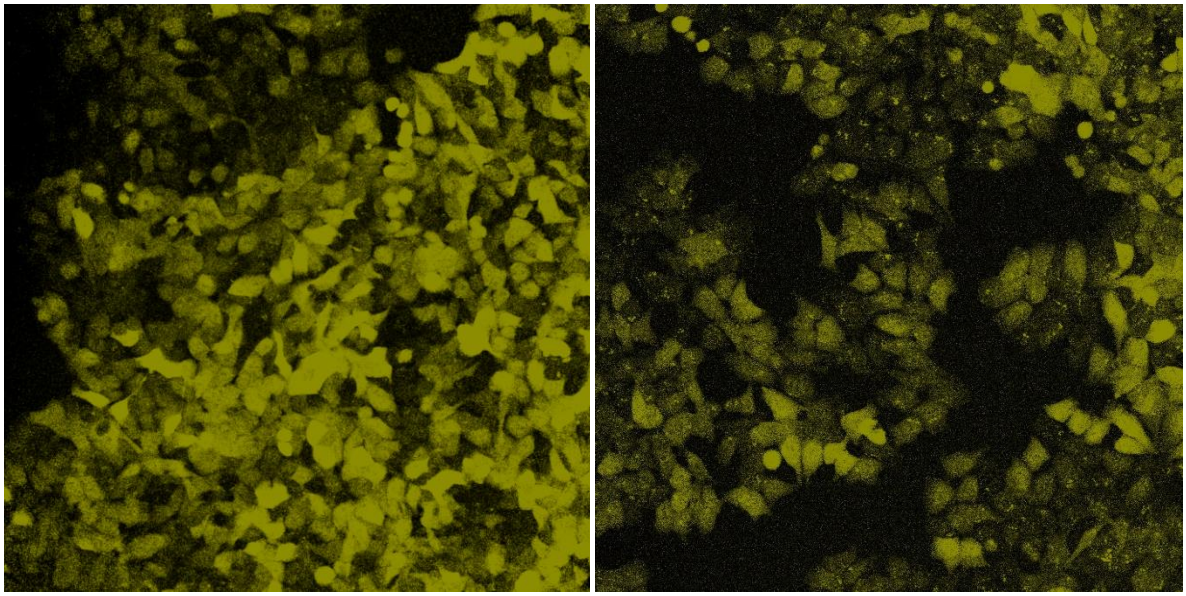


Figure 19. N1-mRNA in Citrate buffer on the left and 5-mRNA in citrate buffer is in the right photo.

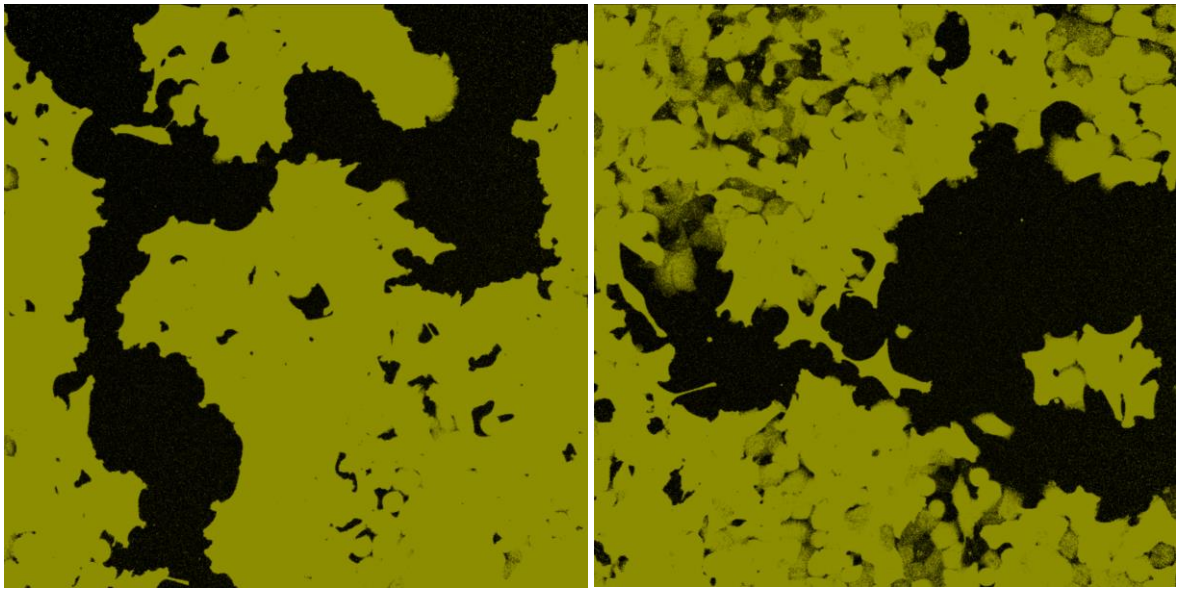


Figure 20. N1-mRNA in acetate buffer in the photo to the left and 5-mRNA in acetate buffer in the photo on the right.

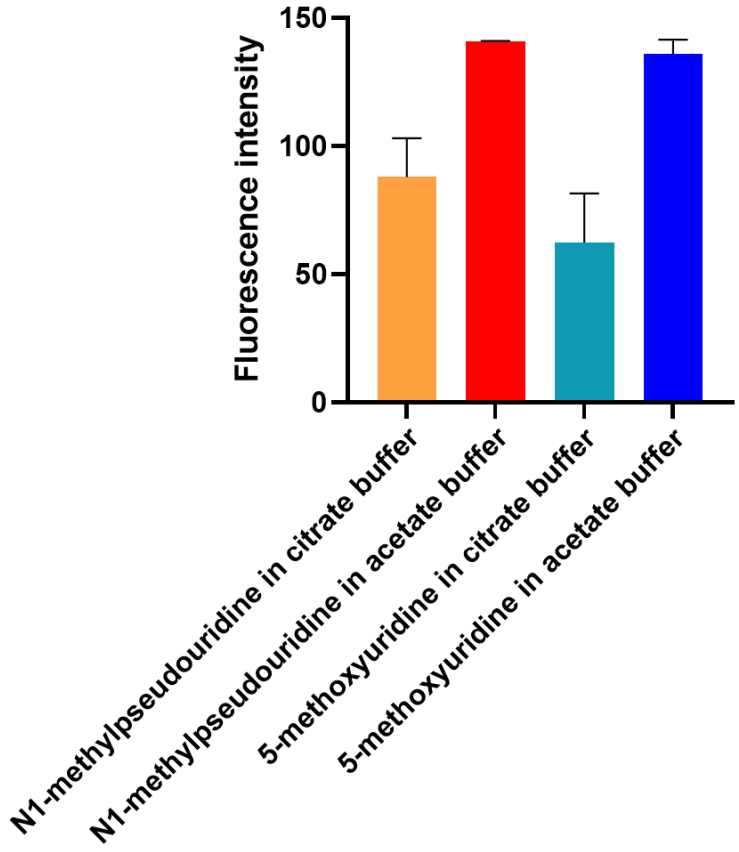


Figure 21. Graph comparing the emitted fluorescent light from N1-mRNA and 5-mRNA in acetate and citrate buffer. (n=16).

It is clear to see that the emitted light from acetate buffer was much higher than the citrate buffer (see figure 19 for citrate buffer and figure 20 for acetate buffer above). The acetate buffer samples however did end up maxing out the light intensity (see figure 20) meaning that the true values of the acetate buffers was even higher compared to citrate buffer than what the above

graph indicates (figure 21), this also explains why the standard deviation of these higher value acetate buffers is so low, because the values could not go higher than 255. Analysing the data in figure 21 showed normal distribution in all but 5-mRNA in acetate buffer. Due to this the non-parametric Mann-Whitney test was performed comparing buffer to buffer and mRNA to mRNA (N1A to N1C, 5A to 5C, N1A to 5A and N1c to 5C) resulting in $p<0.001$ in all cases. We could then confidently conclude that the acetate buffer was better.

To figure out which one was actually stronger of N1-mRNA in acetate or 5-mRNA in acetate, their stacks were taken another time with 10% gain instead of 125%.

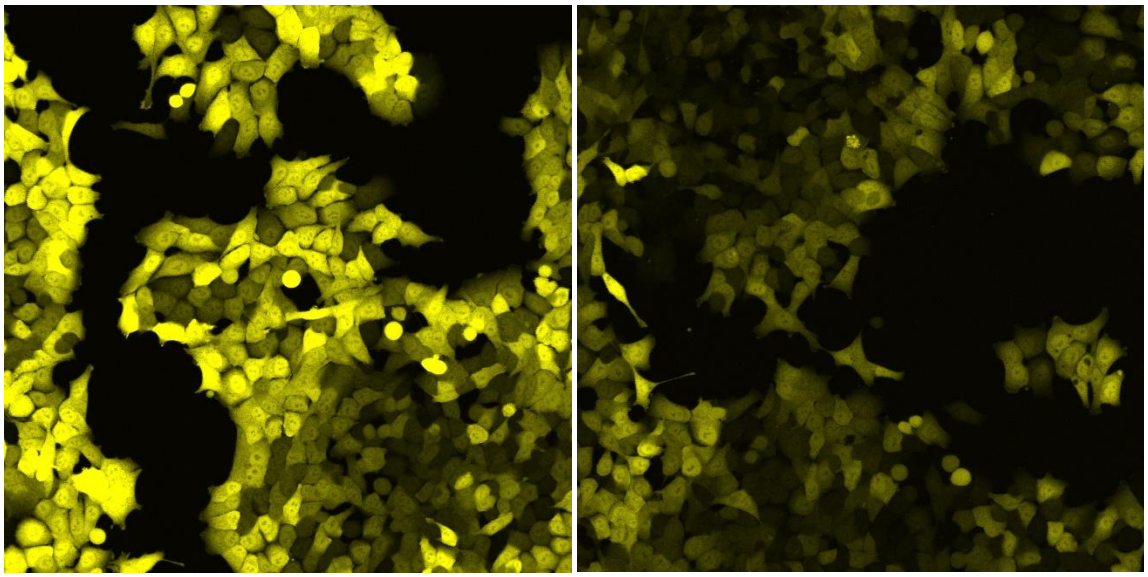


Figure 22. N1-mRNA in acetate buffer is once again on the left and 5-mRNA in acetate buffer is on the right.

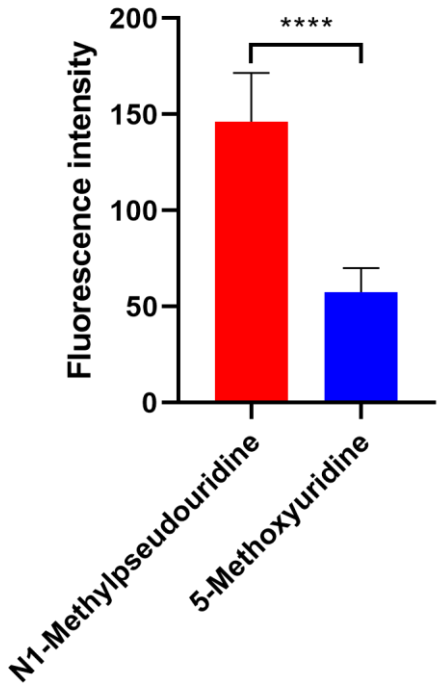


Figure 23. Graph showing the expression of N1A compared to 5A. Statistical analysis showed normal distribution, and unpaired t-test gave p<0.0001, (n=16).

After lowering the signal amplification gain the photos came out much clearer (figure 22) and we could with high confidence say that EGFP expression from N1-Methylpseudouridine was higher than from 5-Methoxyuridine (figure 23).

Discussion

Looking at the results, we were not surprised to see that expression only increased as the LNP concentration went higher. Although when performing a t-test comparing 1:1000 concentration to 1:2000 concentration the P value was 0.15, which is not very convincing, by looking at the images in conjunction with the statistics it was pretty easy to assume that 1:1000 gave higher expression.

In the second experiment we used fresh LNPs, and we saw immediately that using new LNPs gave better expression as the fluorescence with 5-methoxyuridine in acetate buffer now expressed much more. What else we could conclude from this experiment was that acetate buffer was better suited than the citrate buffer, giving much better expression in vitro. We next looked at the best nucleotide modification for maximizing expression using 5-Methoxyuridine and N1-Methylpseudouridine and mixing those mRNAs with acetate buffers. Both expressed extremely well, although statistical analysis showed with high certainty that N1-methylpseudouridne-mRNA gave the most expression. This is an interesting discovery that contradicts the assumption obtained from the manufacturer that 5-methoxyuridine-mRNA would be better suited for in vitro applications, while N1-mRNA was better suited for in vivo applications. Without having tested either in vivo we can at least conclude that N1-methylpseudouridine provides the best expression in vitro.

Conclusion

It is essential to optimize protocols for mRNA expression using LNP and we have found a way to do so:

1. First we tested for ideal LNP concentrations. Finding as expected that the higher concentration of one microliter of LNP solution per mL of cell medium.

2. Then we tested if the buffer used to make the mRNA containing water phase has an effect and we found that a pH 4 acetate buffer was more effective than a pH 4 citrate buffer.

3. Finally we tested if the type of mRNA used makes a difference. We found that using N1-Methylpseudouridine mRNA was significantly more effective than 5-Methoxyuridine in vitro for EGFP expression.

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