Advancing image-based localization of lipid-based nanomedicines for the exploration of targeted drug delivery

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Chapter 1
Introduction

'I may say, indeed, that as yet the investigation of the laws **pertaining to the minute distribution of a chemical substance in the body is only possible when, as in the case of coloured bodies, these are at once recognisable by the eye**. But that it is possible at once **to draw conclusions of therapeutic importance from the laws governing the distribution** was shown in the case of methylene-blue, in which I was able, knowing its distribution in the body, to anticipate for it certain antineuralgic and antimalarial properties which were both established by subsequent investigation.'

- Paul Ehrlich, 1900, *Croonian lecture. – On immunity with special reference to cell life*.

1.1 Lipid-based nanomedicine

Nanomedicines have advanced the field of targeted drug delivery for over the past decades, with fundaments that were built in the early 1870s. The capabilities of dyes that demonstrated high affinities with specific biological structures inspired Paul Ehrlich's belief in targeted therapy.[1] His further interest in immunology and defining the existence of specific receptors received him a Nobel prize[2] which – together with dye specific interactions – provided the fundament for the concept of drug delivery in shape of a "magic bullet".[3] The concept mainly captures the aim of cell specific drug delivery, which targets the diseased tissue and remains harmless to healthy tissue, by avoiding therapy-limiting side effects to increase therapeutic efficacy. Based on the concept of the "magic bullet", many approaches have been made to achieve targeted drug delivery. These approaches comprise a scala of novel designs including nanorobots,[4] light-focused therapy[5] and drug-conjugates.[6] Among these approaches, the nanoparticle in particular, has proven its clinical value over the years.[7] Clinically approved nanoparticle formulations generally comprise colloidal (in)stable particles within a size range of 1 – 200 nm that are bound to/encapsulate an active pharmaceutical ingredient (API), with the ability to achieve a therapeutic effect. Hence, it has been given the name nanomedicine. In most cases, the nanoparticle improves the pharmacokinetic and/or -dynamic properties (*e.g.* biovailability,[8,9] cell uptake,[9] endosomal escape,[10] and reduced toxicity/side effect[9,11]) of the encapsulated API compared to the non-encapsulated API. Although these therapies do not always leave healthy tissue unharmed (e.g. palmar-plantar erythrodysesthesia),[12] nanomedicines have the capability of improving therapeutic potential by shifting drug accumulation towards diseased tissue over healthy tissue.

The nanoparticle has been presented as promising therapeutic in a variety of compositions. The broad scala comprises both inorganic- (*e.g.* polymeric,[13] gold,[14] quantum dots[15]) and organic nanoparticles (*e.g.* mAb conjugates,[16] zein-based,[17] biomimetics,[18] lipid-based[10,19,20]). In specific, lipid-based nanoparticles have advanced from the academic lab bench to the clinic.[10,20] Lipid-based nanomedicines consist out of two main concepts: liposomes and lipid nanoparticles (LNPs). Although there are many more concepts that involve lipid compositions within 1-1000 nm (*e.g.* micelles, cubosomes, disks),[21,22] we here describe liposomes and LNPs for RNA delivery as those have currently resulted in the largest clinical impact and are most relevant for the scope of this thesis.

1.1.1 Liposomes

The first class of lipid-based nanoparticles are liposomes. Liposomes comprise a phospholipid bilayer surrounding an aqueous core and are considered spherical of shape within a predominantly 10-200 nm size range.[23] The phospholipids in the bilayer contain a hydrophilic headgroup and a hydrophobic fatty acid tail.

The composition of the (phospho)lipid headgroup and/or tail has been optimized for its nanoscale assembly, stability and therapeutic potential.[24] Together with other involved components such as buffer and excipients, liposomal formulations can be therapeutically beneficial by altering the biodistribution of the encapsulated API.[20,23]

The discovery of the liposome originates from 1965, when Horne and Bangham discovered membrane-like structures when studying phospholipids under a microscope.[25] It was Gregoriadis in 1971 who suggested the liposome for therapeutic use.[26] After several years of experimentation, liposomal formulations made it to animal studies.[27] Liposomal drug treatment revealed substantial improvement in survival time when compared to non-encapsulated 'free' drug. Use of polyethylene glycol (PEG) allowed for reduced plasma clearance levels[28] and resulted in novel liposome formulations that were circulating longer[29] and improved accumulation into tumour tissue.[30] It was suggested that increased vascular leakage at the tumour site results in tissue-specific accumulation of liposomes, described as the 'enhance permeability and retention effect'.[31] Together with a clever strategy to actively load rigid liposomes with the chemotherapeutic doxorubicin,[32] first clinical application[33] of doxorubicin liposomes (Caelyx/Doxil) has resulted in treatments for ovarian cancer, AIDS related Kaposi's sarcoma, and multiple myeloma.[20]

Liposomal formulations have made clinical successes in fields of oncology and beyond. Encapsulating API's such as doxorubicin resulted in a major drive for other labs and institutes to explore its potential in the field of drug delivery. Next to cancer therapy, liposomal formulations have been tested for use as vaccines, photodynamic therapy, fungal diseases, and analgesics.[34] In view of nucleic acid delivery, where oligonucleotide-based therapeutics are delivered to cells and tissues of interest, the liposomes showed potential as carriers with cationic lipids and properties that would allow for cellular uptake,[35] however, also revealed toxicity related to use of such cationic lipids.[36,37] Although liposomes were expected to make clinical advances in gene delivery,[38] it was the LNP that brought nucleic acid-based therapies to the clinic.

1.1.2 Lipid nanoparticles for nucleic acid-based therapy

Lipid nanoparticles are the most recent type of lipid-based nanoparticles achieving major clinical success. LNPs display a similar size range as liposomes, predominantly within 10-200 nm. Different from the liposome, the LNP has a solid core that consists of lipids and excipients, and can be loaded with oligonucleotide-based structures upon binding with ionizable lipids. Incorporation of PEGylated lipids on the outer lipid leaflet has been considered to stabilize the formulation by preventing fusion.[39] Cryo-transmission electron microscopy (cryo-TEM) has revealed many sub LNP morphologies, including ones with a lipid monolayers,[40] -bilayers,[41] 'bleb-structures',[42] (poly)amorphous

and lamellar lipid packing,[43] and/or those suggested to be phase-separated.[44] Therefore the LNP subtypes and suggested schematic morphologies display a relatively complex system compared to the liposomes. Unlike its morphologic complexity, the foundations of the LNP required only two key developments that have propelled its clinical success for oligonucleotide delivery: nucleoside base modification and the use of the ionizable lipid.

It was the discovery of mRNA in the period 1947-1961,[45] that provided the foundation for its worldwide therapeutic efficacy during the recent COVID-19 pandemic. Upon the discovery, further developments in the 1980s demonstrated that mRNA could be *in vitro* transcribed, which led to many proof-of-concept animal studies in the 1990s and until today, optimized formulation designs to improve mRNA encapsulation, stability, delivery, cellular uptake and endosomal escape.[46] It was in 2005 that, based upon the findings that *in vitro* transcribed mRNA revealed to result in inflammatory signalling, Kariko and Weissman demonstrated the use of nucleoside modified RNA,[47,48] providing more foundations for oligonucleotides to have improved therapeutic potential by lowering their immunogenicity. Just as important as the reduced RNA immunogenicity was the design of the formulation that would unlock its therapeutic potential: the ionizable lipid nanoparticle.

LNPs allow for oligonucleotide delivery in which the ionizable lipid allows for controlled encapsulation and release of nucleic acid-based cargo. Compared to the approach of liposomal delivery where cationic bilayer lipids bind electrostatically with nucleic acid-based cargo on its membrane, the LNP fundamentally differs by encapsulating most of the cargo internally with use of ionizable lipids.[49,50] The LNP formulation is assembled by using microfluidics. Here, lipids that are cationic in charge at acidic pH (~4.0) allow for complexation with the anionic phosphate groups of oligonucleotides that – together with other stabilizing (phospho)lipids, cholesterol, PEGylated lipids and excipients – can form stable LNPs in buffer at physiological pH (~7.2). When exposed to and taken up by cells, LNPs can end up in lyso- and endosomal compartments, where the pH drops often below the pKa of the ionizable lipid, resulting in the protonation of the ionizable lipid. As a consequence, cationic lipids can have electrostatic interactions with anionic endosomal membranes, which may lead to the destabilization of the LNP and the cytosolic release of its oligonucleotide-based cargo.[51]

After cargo release from the LNP, the nucleic acid-based therapeutic in disguise of siRNA could be used to suppress gene expression. It was patisiran (ONPATTRO) that pioneered LNP-mediated oligonucleotide delivery for the treatment of hereditary transthyretin (TTR) amyloidosis.[10] Intravenous injection of liver-targeted LNPs resulted in siRNA delivery that anticipated TTR production by binding to its instructive mRNA, preventing the formation of amyloid deposits. Not only intravenous (IV)-administered and siRNA-based, but

other routes of administration with differing nucleic acid-based cargo demonstrated clinical potential of the LNP. Here, cargo in the form of mRNA can be transcribed into an antigen/functional protein. The COVID-19 pandemic propelled the clinical use of the LNP by urgently needing a vaccine, where the LNP was explored for its opportunity to present the antigen with the use of mRNA delivery. In contrast to prior vaccine technology where the formulation encompassed the viral antigen within its formulation, the LNP allowed for the *in vivo* production of the antigen. The COVID-19 vaccine was introduced to the tissue by intramuscular administration, resulting in the cytosolic release of mRNA to its surrounding antigen presenting cells, and subsequent antigen presentation to obtain a controlled immune response. This has led to a clinical success for LNP as a vaccine of which two formulations (*e.g.* Moderna and BioNTech/Pfizer) were considered most prominent during the COVID pandemic of 2019-2023.[52,53] Together with the scalability of mRNA production platforms and the short-lived mRNA presence in the cytosol, the LNP technology presented itself as a powerful vaccine.[53] Importantly, the versatility of the LNP has unlocked a variety of novel oligonucleotide-based therapies.

1.2 Targeted lipid-based nanomedicine

Site-specific administration of lipid-based nanoparticles is the most straightforward way of achieving targeted delivery. By physically changing the site of exposure to the formulation, one could be able to achieve a local therapeutic effect. Current advancements have already demonstrated a wide variety of exposed sites for achieving a local therapeutic effect, in specific for LNP delivery.[54] Here, the oral route has been explored for the treatment of inflammatory bowel disease,[55] lungs by inhalation of LNPs for the treatment of a viral infection,[56] muscular tissue for the treatment of Duchenne disease,[57] intracerebral injection the treatment of orthotopic glioblastoma,[58] microporation of the skin for LNP delivery,[59] and gene editing in the corneum.[60] Nonetheless, the most common route remains IV administration due to systemic tissue accessibility, predictive and controlled dosing and limited safety risks. Despite promising advances for ONPATTRO, clinical translation for IV-administered targeted lipid-based nanomedicine beyond the liver remains a challenge.[61]

Both liposome and LNP technologies eagerly await novel advancements that can aid in improved targeted therapeutics via IV administration. The development of novel lipid-based nanomedicine requires the understanding of both fundamental pharmaceutical principles for the API[62] as well as advanced nanoparticle properties[63] (*e.g.* particle number, morphology), under the notion that involved lipids could function both as APIs and/or as excipients.[64] Having an in-depth view of all components involving the lipid-based nanomedicine would not only be useful to meet regulatory criteria, but could also be advantageous for the design and optimization of targeted therapy. Lipid compositions have already shown to be of importance for targeted delivery when administered IV.

Exemplary, ONPATTRO revealed composition-specific adsorption of apolipoprotein E on the LNP surface that resulted in improved delivery towards the hepatocytes.[65,66] More promising work based solely on lipid composition screening has been presented. Although its mechanism of biodistribution remains unknown, Dahlman has demonstrated with a high-throughput approach to identify promising formulations for increased tumour delivery among 96 formulations that solely differed in lipid composition.[67] Another screening (128 formulations) demonstrated the importance of lipid composition on safety, which suggests that stereopure lipids can be more tolerant compared to racemic mixtures.[68] A change in lipid composition alters not only its biodistribution and/or safety, but also particle morphology and organization, which could be of indirect interest in the design of targeted lipid-based nanomedicine. Patel et. al. demonstrated that the use of naturally occurring sterols could change LNP efficacy with a suggested role for the LNP morphology that remains to be further explored.[69] Moreover, morphologic LNP 'bleb' structures have been suggested to play a role in mRNA stability that could enhance its potency.[70] Although it remains challenging to correlate indirect evidence (*e.g.* morphology and protein binding) in the direction of targeted delivery, what stands out is that targeted lipid-based nanomedicine should be addressed as whole lipid systems, in which each lipid (and its enantiomer) can be advantageous in its exploration for therapeutic potential.

1.3 The zebrafish embryo for the investigation of IV-administered targeted lipid-based nanomedicine

By providing cellular insights upon IV administration, the zebrafish embryo can aid in improved understanding of diverging *in vivo* outcomes of lipid-based nanoparticles. With its optical transparency and options for genetic manipulation as key features, the zebrafish embryo has been considered a unique model for novel *in vivo* insight into nanomedicine.[71,72] The model is complementary to cell culture systems, which lack blood circulation and have limited multicellular complexity, as well as to larger laboratory animal species with confined real-time microscopic insight. Exemplary, the transparent appearance of the zebrafish combined with knock out models for the stabilin receptor provided crucial observed evidence on the involvement of scavenger receptor Stabilin-2 in the biodistribution of fluorescently labeled nanoparticles.[73] The zebrafish also allowed for rapid observation on the impact of lipid composition on fluorescently labeled liposomes. Here, the impact of phospholipid fatty acid tail length has been addressed, which revealed changes in liposome morphology, but also in clearance pattern.[74] The model allowed Papadopoulou et. al. to discover the cell-specific delivery of phase-separated liposomes towards the zebrafish embryonic brain endothelium, in which liposomes' composition and 'parachute morphology' are suggested to play an important role.[75] Also surface charge has been suggested to play an important role in targeting lipid-based nanoparticles.

Pattipeiluhu et. al found that by switching from DSPC to DSPG in LNP compositions resulted in improved hepatocyte delivery in mice,[76] whereas Endotag-1 has been suggested to accumulate to nearby vascular endothelial cells.[73] Moreover, innovative approaches including a proof-of-concept study for photoactivated targeting of liposomes *in vivo*, in the zebrafish have demonstrated their unique value. Here, Arias-Alpizar et. al. found that light-triggered photo-switching lipid moieties could result in site specific accumulation of rhodamine labeled lipids.[77] Together, screening of lipid-based nanomedicine in zebrafish allows for rapid observations *in vivo* upon IV administration which can aid in exploring novel targeting approaches.

1.4 Localization of lipid-based nanoparticles in zebrafish

Confocal fluorescence microscopy is widely used for the localization and biodistribution of lipid-based nanomedicines in zebrafish embryos.[71-88] By localizing the emission of a fluorophore in a three dimensional spatial context, confocal microscopy neatly supports the investigation of fluorescently labeled nanomedicine in the transparent zebrafish embryo model that can have fluorescently labeled cell types. Here, the correlation between lipid-based nanomedicine and cell specific structures can provide novel insights on its cell/tissue specific targeting. Other methods used to localize lipid-based nanomedicine in zebrafish embryos are promising, but currently limited. For example, a recent study revealed the subcellular organization and quantification of silica-based nanoparticles *ex vivo* with the use of cryo-TEM.[89] Nonetheless, lipid-based nanoparticles remain hard to visualize and identify *ex vivo* solely based on electron density. In view of profiling the biodistribution and pharmacokinetics of IV-administered medicine in zebrafish, van Wijk et. al. developed a methodology that allows for nanoscale blood sampling from zebrafish embryos,[90] which has the potential to be highly beneficial to determine the stability, clearance profiles and metabolite formation of lipid-based nanomedicine. Although promising, van Wijk also emphasized that blood sampling is currently limited by its practicalities and is awaiting an automated approach. Next, fluorescence-activated cell sorting (FACS) has proven to be of value for perceiving insight into the cellular distribution of lipid-based nanomedicine, where fluorescently labeled nanomedicine can be correlated with specific cell types to determine its uptake levels.[91] The group of Dahlman used this approach to plot the cell specific distribution of LNPs.[92] These LNPs contained a DNA 'barcode' that allowed to analyse cellular uptake among differing LNP batches, when processed and analysed by FACS and DNA sequencing. Studies have been performed in *in vitro* cell culture setups, as well as in mice and require washing of the medium and/or perfusion of the blood circulation to prevent false positives due to passive distribution *ex vivo*. Currently such studies are not possible to perform in zebrafish embryos, as the required washing and/or perfusion methodology for this model has yet to be

developed. The status quo is that confocal fluorescence microscopy is currently the most relevant technology for the localization and distribution of lipid-based nanoparticles in zebrafish.

Confocal fluorescence microscopy also has its shortcomings and limitations for investigating lipid-based nanomedicine in zebrafish embryos. First, the outcome of confocal microscopy imaging is intricately linked to the fluorescent source integrated into or onto lipid-based nanoparticles, thereby making it susceptible to misinterpretation when the source dissociates from the nanoparticle. Fluorescent reporter molecules are crucial for understanding the localization of lipid-based nanomedicine in zebrafish. Although encapsulation of a fluorescent molecule can give insight into the biodistribution of lipid-based nanoparticles, leakage of the probe could also lead to undesired false positive results.[93] As an alternative, most studies make use of lipid-fluorophore conjugates (LFCs). The LFC is known to be embedded in a lipid bilayer when present in buffer[94] and has been widely used as a reporter molecule for lipid-based nanomedicine in zebrafish embryos,[71-78,80-86] but also in cell culture systems[95-99] and animals studies.[100-105] Interestingly, recent work has demonstrated that when lipid-based nanoparticles – formulated with an LFC – are exposed to a biological environment, the LFC can dissociate.[106] Based upon this knowledge, it is plausible that LFC-labeled liposomes upon injection in the zebrafish embryo perform similar dissociative phenomena. As the analysis of lipid-based nanoparticles in zebrafish is mainly based on the interpretation of LFC localization, it is of crucial importance that they remain attached when injected to provide accurate localization.

Secondly, confocal microscopy remains resolution-limited for the investigation of subcellular processes involved with targeted lipid-based nanomedicine. The diffraction limit, described by Ernst Abbe in 1873,[107] confines the resolving capabilities of a fluorescent confocal microscope to ~200 nm. As a result, microscopic localization of fluorescently labeled lipid nanoparticles has a diffraction-limited uncertainty, which limits the spatial analysis on subcellular compartmentalization occurring at the nanoscale (< 200 nm) level (*e.g.* endo-/lysosomal uptake and release), protein binding (*e.g.* adsorption, receptor) and cell uptake (*e.g.* brain endothelium). Approaches have been made that successfully surpass the diffraction limit by technologies such as STED-[108] and STORM microscopy,[109] however they are colour-limited and work with limited tissue thickness (<130 µm).[110] The recent introduction of 'expansion microscopy' could be a game changer that can provide localization beyond the diffraction limit in thick tissue (500 µm). By physically expanding hydrogel-embedded tissue, expansion microscopy (ExM) allows for super-resolved imaging.[111] Briefly explained, tissue of interest can be anchored into a hydrogel which can be subsequently expanded upon denaturing and/or digesting treatment and washing with ddH_2O. The technology has a low accessibility level and can

be easily introduced to so-called 'beginners'.[112] Further developments have progressed in ExM towards higher expansion factors (10X)[113] and compound specific imaging.[114,115] Moreover, ExM has recently been utilized for high resolution visualization of the brains- and whole tissue of zebrafish embryos.[116,117] These recent developments in ExM lay the groundwork for investigating lipid nanomedicine localization in zebrafish beyond the diffraction limit.

Overall, confocal fluorescence microscopy is a crucial tool for the localization of lipid-based nanoparticles within zebrafish embryos. However, challenges persist for improving fluorescent labeling and pushing the boundaries of resolution limits. Overcoming these hurdles can be advantageous in obtaining advanced understanding for designing lipid-based nanomedicines for targeted delivery.

1.4 Aim and outline of the thesis

Microscopic insight on lipid-based nanomedicine *in vivo* remains limited to the perception of the knowledge that could be obtained: if we interpret only what we see, then we only believe to know. Although 'believing to know' encapsulates an undefined amount of uncertainty in the exploration of lipid-based nanomedicine, it remains a fundamental requirement to draw hypotheses that could result in major scientific breakthroughs. Knowing the distribution of methylene blue within the body allowed Ehrlich to anticipate on exploring its therapeutic potential for antineuralgic and antimalarial use.[118] In this light, by unravelling the specific locations of lipid-based nanomedicine upon screening of lipid composition, one could design its targeted therapy. Recent work underscores this concept by demonstrating that the evaluation of LNP localization in zebrafish embryos resulted in rationally designed LNP-mRNA delivery to the hepatic reticuloendothelial system in mice.[43]

The aim of this thesis is to advance the image-based localization of lipid-based nanomedicine, which could aid in improved understanding of targeted drug delivery. I want to achieve this by providing fundamental knowledge on the truthfulness of the source and the location of lipid-based nanomedicine and its *in*- and *ex vivo* environment.

Chapter 1 provides a brief perspective of lipid-based nanomedicine on targeted drug delivery, the role for the zebrafish embryo, and its fluorescent localization *in vivo*.

Chapter 2 describes criteria for the use of fluorescent reporter molecules for lipid-based nanomedicine, LFCs. Recent insights on possible LFC dissociation from lipid-based nanoparticles when injected into zebrafish motivated me to investigate the truthfulness of the fluorescent probe in relation to its *in vivo*

outcomes. The use of LFCs can be highly beneficial for the localization of lipid-based nanomedicines, however its fatty acid backbone and headgroup must be taken into account to remain embedded in the lipid-based nanoparticle to provide predictive localization.

Chapter 3 illustrates the use of LFC-labeled LNPs to screen brain endothelium specific targeting in zebrafish embryos prior to transfection. Insights from previous work on the incorporation of DOaG were screened for second generation targeted nanomedicine, LNPs. We demonstrate the targeting and transfection of the brain endothelium in zebrafish embryos upon IV administration. The work highlights the need for targeting prior to transfection and imposes perspective on brain endothelium transfection strategies.

Chapter 4 describes a protocol to obtain hydrogel-expanded embryonic zebrafish brains to have a nanoscale look at the events of nanomedicine at blood-brain barrier (BBB). The BBB is the gatekeeper of drug delivery towards the central nervous system and remains a challenge for IV-administered drug delivery towards the brain. Routes for active targeting (e.g. endo-, para-, and/or transcytosis) are explored; however, complex barriers require in-depth analysis. Adding a spatial component to the analysis could yield a better understanding for targeted delivery. With the use of a novel ExM technology we aimed to physically expand whole zebrafish embryo brains and the BBB, including the stabilization and anchoring of lipid-based medicine *ex vivo*.

In **Chapter 5** we describe a plate model for expansion microscopy. In Chapter 4 we appreciated the use of ExM. Nonetheless, we hypothesized that providing a plate model of this technology would make it accessible for any type of researcher, as well as provide a starting point towards standardization and automation. In fact, during our studies the need for an ExM platform[119] in form of a well plate[120] has confirmed in the literature. With the use of a simple setup we present a technology that would enable many researchers to explore ExM in an accessible manner.

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