Towards a mechanistic understanding of nanoparticle behavior using zebrafish

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Chapter 1

General Introduction & Scope of the thesis

Image: Fluorescently labelled nanoparticles (cyan) intravenously injected in a zebrafish embryo. Endothelial cells (green) showing the intersegmental vessels and dorsal aorta.

1.1 Nanomedicine at a glance

Nanomedicine refers to the application of nanotechnology in the development of devices and therapeutics to benefit diagnosis and/or treatments.[1] This field has been motivated by the concept of targeted drug delivery, which intends to enhance the therapeutic efficacy and safety profile by protecting the cargo (*i.e.* drugs, genetic material) from external degradation, controlling its delivery, and thereby helping to minimize the side effects.

Due to their various characteristics, nanoparticles are believed to be suitable systems for different commercial applications[2] from sensing,[3] detection of proteins,[4] imaging,[5] to vaccine development,[6] and drug/gene delivery[7-10] purposes. In the last ~50 years a myriad of materials and techniques have been applied to the design, characterization, and production of different types of nanosystems. Despite the variety of applications, it can be surprising that commercialized products are very limited, suggesting that nanoparticles are not 'the magic bullet' that were initially expected to be. This urgently called for further investigation, or a change in the investigation approach to be performed, and maybe a reassessment in the capabilities of nanoparticles.

The development of more efficient nanoparticles will definitely contribute to the clinical success. However, it requires a thorough understanding of the nano-systems used and their performance *in vivo*. Mechanistically, nanoparticles mimic the behavior and uptake of natural agents, such as viruses, to deliver a cargo. Viruses are known to enter the body, travel in the bloodstream, find the target tissue and be taken up by the cell. Subsequently, degradation of the "shell" and release of the cargo occur.[11] Furthermore, the immune system recognizes both viruses and nanoparticles as intruders and tries to remove them from the organism to maintain homeostasis. In other words, similar to viruses, nanoparticles need to pass different barriers to reach the intended place; these series of events can only be observed simultaneously in a living organism, where all the organs and systems are present and dynamic interactions take place. In this respect, translation of nanomedicines (see page 14-16) is one of the greatest barrier to the clinics, therefore models that help to understand the fundamental behavior *in vivo* of such particles are required.

Classification of nanocarriers

Depending on the material used for their preparation, nanoparticles can be classified into two main groups: organic and inorganic. The first class includes biodegradable and non-toxic materials such as polymeric dendrimers (*i.e.* micelles, vesicles), hydrogels, proteins and lipid-based delivery systems. The inorganic group consists mainly of non-toxic metal-based particles (*e.g.* gold, aluminum, zinc), coated quantum dots, and mesoporous silica. In this classification, the nanoparticle type that has demonstrated the highest success in the clinic and commercialization is the lipid-based nanoparticles. Therefore, they are described and used as research platforms in this thesis.

Lipid-based nanoparticles comprise the most well-studied systems for drug delivery and one of the most commonly used strategies in nanomedicine. Their characteristics of biocompatibility, biodegradability, low toxicity, and ease of production, makes them versatile and attractive materials for drug delivery.[12] Three main groups can be mentioned as lipid-based nanoparticles: lipid micelles, liposomes, and lipid nanoparticles (LNPs). Micelles are simple structures formed by amphiphilic lipids (preferably of conical shape) in an aqueous solution that, above the critical micelle concentration, self-assemble into a micellar system with a hydrophobic core.[13] They are characterized by a high dynamic inherent nature and therefore, are found mostly combined with polymer blocks as delivery systems.[14] In contrast to micelles, liposomes and more recently LNPs have demonstrated more versatility by reaching clinical applications.[15] Liposomes are composed of amphipathic lipids that, mimicking biological membranes, self-assemble into spherical vesicles forming a bilayer and an aqueous core (**Figure 1**). This feature allows entrapment of hydrophilic or lipophilic compounds, protection of the cargo against degradation and lowers their apparent toxicity. To achieve targeting, the outer layer of the lipid-based nanoparticles can be functionalized by coupling of ligands, antibodies, polymers, proteins, *etc*. thereby tuning the *in vivo* behavior. Characteristics like rigidity is mainly dependent on the acyl/alkyl chain length and saturation, while surface charge is defined by charges present in the lipid head group. These properties, together with size and stability, will also affect pharmacokinetics of liposomes *in vivo* and subsequent interactions with cells. Hence, the physicochemical properties can be used to tune lipid-based nanoparticle *in vivo* fate and cellular uptake.

Some of the challenges that have been overcome with liposomes as a delivery platform in diagnostics and clinics are: reduction of toxicity, improvement of the

therapeutic index of delivered drugs,[9] protection of sensitive materials (*e.g.* DNA, RNA, oligonucleotides, cytosine arabinose) from early inactivation or degradation,[16, 17] and enhancement of the pharmacokinetic profile.[10, 18] Gene delivery, however, is more difficult to achieve due to limitations in the stability, safety and delivery of nucleic acids. This modality aims to regulate gene expression by successfully delivering nucleic acids to a particular tissue of interest. Hence, downregulation of genes could be achieved by small interfering RNA (siRNA), microRNA (mRNA) or antisense oligonucleotides (ASOS). In contrast, increase and correction of the gene expression are mediated by messenger RNA (mRNA), small activating RNA (saRNA), DNA, splicing-mediated ASOS or as a result of the use of a more complex gene technique, clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISR/Cas9).[19]

Nucleic acids have been chemically modified to solve the challenging tasks of stability and reduction of side effects, for example, synthetic RNA molecules were modified to regulate immune responses and suppress inflammatory responses.[20-22] From the nanotechnology perspective, development of non-viral vector based delivery systems contributes in the protection of the cargo to circumvent some of the biological barriers and ensure the biological activity of the nuclei acid-based drugs once target site is reached. Encapsulation of nucleic acids into the core of a nanocarrier prevents intravascular degradation (*e.g.* breakdown by serum nucleases) and rapid renal clearance due to its small size. Furthermore, due to the negative charge and hydrophilic nature, naked nucleic acids have a poor ability to cross cell membranes. Thus, nanocarriers are required to promote endocytosis leading to cargo delivery in the cytosol (RNA) or the nucleus (DNA) of the targeted tissue. Intracellular barriers, like endosomes, also limit the efficacy of the desired therapy. Consequently, strategies to overcome this barrier successfully result in the development of cationic lipids that facilitate the entrapment of DNA/RNA and promote endosomal escape.[23-25] Incorporation of these lipids has led to the development of a new generation lipid-based nanoparticles, known as lipid nanoparticles (LNPs).

LNPs were developed as an strategy to deliver nucleic acid-based therapeutics and to improve the encapsulating capacity of lipid-based delivery systems. Compared to liposomes, LNPs are more complex solid lipid-based nanoparticles with a monolayer outer membrane and a non-aqueous hydrophobic core.[26] Typically, LNPs incorporate at least four components: helper lipids (*i.e.* cholesterol and phospholipids), ionizable lipids (*i.e.* DLinDMA, DLinKC2DMA,

DLinMC3DMA), the genetic material of interest (*i.e.* DNA/RNA), and a polyethylene glycol (PEG) lipid.[27, 28] Structurally, cholesterol, the helper lipid and the ionizable lipid in complex with the genetic material form a lipid core, coated by a pegylated surface (**Figure 1**). Rapid mixing of these components in an ethanol solution with an aqueous buffer (at pH 4), in a 1:3 ratio, is normally achieved employing microfluidic systems.[26] In that way, and commonly in a nitrogen to phosphate ratio (N/P) of ~6, efficient entrapment of the genetic cargo can be reached (>50 %), quantified by using a fluorescent dye (*e.g.* RiboGreen assay).[29] This results in a homogenous population of small LNPs (<100 nm), with a solid complex core and a surface rich in helper and PEG-lipids.

As a gene delivery system, the *in vivo* efficacy of the LNP relies mainly on the delivery efficiency and the stability of the cargo; whereas the potency of such a system firmly depends on the ionizable and PEG-lipids used.[30-32] The ionizable lipid is an essential component in the encapsulation of LNPs, as it is cationic at low pH (*e.g.* pH 4). This allows association between the negatively charged nucleic acids and the cationic lipid mediated by electrostatic interactions, upon mixing. In addition, once the LNPs are internalized in the cytoplasm, the acidic pH of the endosomes induces protonation of the ionizable lipid resulting in membrane destabilization and consequent release of the cargo, promoting a required endosomal escape.[33] The PEG-lipid primary function is to support the self-assembly; however, it also influences the nanoparticle size and the dissociation rate.[30, 31] Size of LNPs depends on the amount of lipid-PEG during assembly, increase in PEG-lipids concentration leads to decreased LNP size. For instance, incorporation of 1 and 5 mol% results in LNPs with a diameter of 44,5 and 22,4 nm respectively.[31] Furthermore, since the PEG-lipid resides preferentially on the outer monolayer of the LNP, its rate of dissociation *in vivo* impacts the release of the cargo and the activity of the nanosystem.[32]

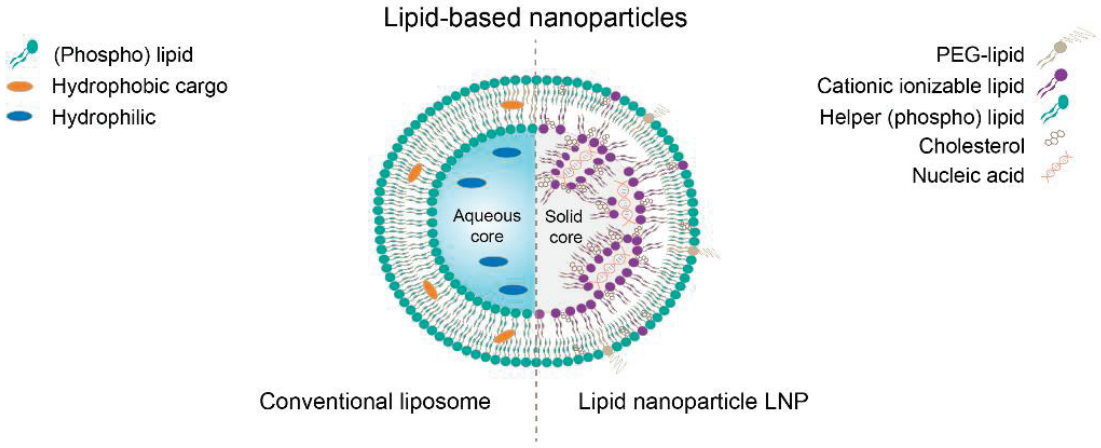

Figure 1. Lipid-based nanoparticles platforms. On the left side, schematic representation of a conventional liposome composed of lipid molecules with a hydrophilic head group and a hydrophobic tail that self-assemble to form a bilayer containing an aqueous core. This characteristic allows the encapsulation of hydrophobic or hydrophilic cargos. On the right side, a lipid nanoparticle (LNP) formed by a combination of lipids (cationic ionizable, helper, PEG) entrapping nucleic acids in a solid (non-aqueous) core.

Clinically approved (lipid-based) drug delivery systems

The potential of the lipid-based drug delivery systems has been demonstrated by delivering anticancer agents, antibiotics, combinations of drugs, and many more. Clinically approved lipid-based nanoparticles are reviewed in reference [15]. Although different routes have been used in the administration of approved formulations, the intravenous (*i.v.*) application is the most commonly used route. Approved liposomes *i.v.* administered are summarized in **Table 1**.

The first liposomal formulation approved (in Europe) in the 90s was Ambisome®,[34] containing amphotericin B, an antibiotic used against acute fungal infections. Later, two similar formulations (Abelcet®[35] and Amphotec®[36]) were approved to treat aspergillosis. Besides fungal infections, drugs loaded liposomes were developed to treat various forms of cancer. Liposomal formulations such as Myocet®,[37] Doxyl®,[18] LipoDox®,[38] DaunoXome®,[39] Vyxeos®,[40] Marqibo®[41, 42], and Onyvide®[43] were approved due to prolonged circulation times and tumor extravasation of the entrapped and protected cytotoxic drugs: doxorubicin (*i.e.* breast, ovarian cancer), daunorubicin and cytarabine (*i.e.* Kaposi's sarcoma), vincristine (*i.e.* acute leukemia) and irinotecan (*i.e.* adenocarcinoma of the pancreas). In 2012, one more formulation was approved, Mepact®, and commercialized for the treatment of osteosarcoma associated with chemotherapy. These liposomes deliver mifamurtide, which

Table 1. Clinically approved *i.v.* administered liposomes.
(Adapted from references [44, 45]).

Formulation	Lipid composition	Cargo	Indication
Ambisome®	DSPC, DSPG, Cho (2:0.8:1)	Amphotericin -B	Fungal infections (*i.e.* aspergillosis)
Abelcet®	DMPC, DMPG (7:3)		
Amphotec®	Cholesteryl sulfate		
Doxyl®/ Caelyx® LipoDox®	HSPC, Cho, PEG2k-DSPE (56:39:5)	Doxorubicin	Kaposi's sarcoma, breast and ovarian cancer
Myocet®	POPC, Cho (2:1)		Breast cancer
DaunoXome®	DSPC, Cho (2:1)	Daunorubicin	Kaposi's sarcoma, leukemia
Vyxeos®	DSPC, DSPG, Cho (7:2:1)	Daunorubicin + cytarabine	Acute myeloid leukemia
Marqibo®	Sphingomyelin, Cho (60:40)	Vincristine	Acute lymphoblastic leukemia
Mepact®	DOPS, DOPC (3:7)	Mifamurtide	Osteosarcoma
Onyvide®	DSPC, Cho, PEG-2K-DSPE (3:2:0.015)	Irinotecan	Adenocarcinoma of the pancreas
Visudyne®	DMPC, EPG (1:8)	Verteporfin	Age-related macular degeneration, myopia, ocular histoplasmosis

Abbreviations: Cho, cholesterol; DMPC, 1-α-dimyristoylphosphatidylcholine; DMPG, 1-α dimyristoylphosphatidylglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3- phosphatidylserine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; HSPC, hydrogenated soy phosphatidylcholine; DSPG, 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol; EPC, egg phosphatidylcholine; EPG, egg phosphatidyl-glycerol; PEG 2k-DSPE, polyethylene glycol 2000-1,2-distearyl-*sn*-glycero-3-phosphati-dylethanolamine; POPC, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine.

binds to toll-like receptors 4 stimulating an immune response mediated by macrophages and generating an antitumor activity.[46] In a different context than cancer, the liposomal formulation containing the photosensitizer verteporfin, was developed to decrease the risk of vision loss.[47] Visudyne® approved for photodynamic therapy to treat subfoveal choroidal neovascularization due to age-related macular degeneration.

LNPs have only recently obtained approval as a drug delivery platform, **Table 2**. Onpattro® (Patisiran) is the pioneer in the LNPs-based RNA therapeutics. Approved in 2018, Onpattro® uses small interfering RNA (siRNA) to treat polyneuropathies caused by a genetic disease named amyloidogenic transthyretin (TTR) amyloidosis.[33] This treatment silences the TTR mutated gene by delivering siRNA to hepatocytes. Targeting of this liver cell type is mediated by the binding of apolipoprotein-E to the cell surface which subsequently guide the LNPs to the low-density lipoprotein receptor (LDLR) family members.[48] Other LNP siRNA-based therapies have reached the clinical trials;[49] for instance TKM-080301 is a formulation for solid tumors or lymphomas. The antineoplastic activity of this formulation, which silence the overexpression of polo-like kinase 1 (PLK1) in tumor cells, was demonstrated so far in xenograft models and preclinical studies.[50, 51]

In addition, the great potential of LNPs has been demonstrated recently in the development of non-viral vaccines[52] due to its ability to deliver vaccine antigens and express proteins upon cytoplasmic mRNA delivery. In this context, LNPs technology is under investigation of various immunization approaches against viruses (*e.g.* influenza,[53] Zika virus,[54] HIV-1[55], cytomegalovirus[56]) and for cancer immunotherapies.[57] Compared to conventional vaccine platforms, using live attenuated and inactivated viruses, LNPs not only decrease the risk of potential infection, but also offers the possibility to be applied in non-infectious diseases (*e.g.* cancer). Moreover, the rapid development and manufacturing scalability are exemplified by the recent approval (in emergency cases by FDA) of mRNA-based vaccines for the prevention of the pandemic COVID-19, highlighting the versatility of this technology. Comirnaty® (also called BNT162b2)[58] and Moderna COVID vaccine®[59] (mRNA-1273) encode a mutant viral spike (S) protein of SARS-CoV-2. These LNPs formulations are used as a carrier of the mRNA to facilitate efficient intracellular delivery and to avoid extracellular degradation by RNAses.[60] After intramuscular injection, LNPs are taken up by cells via endocytosis and ultimately mRNA is delivered in the cytosol. As a result, the S protein is produced and translated into a functional protein to finally induce the adaptive immune response and formation of antibodies against the

virus. Due to the ease of design, LNPs technology promises a more personalized genetic therapy for the future, where a large variety of diseases can be effectively treated.

Table 2. Clinically approved LNPs (December 2020).

Formulation	Lipid composition	Route of administration	Cargo	Indication
Onpattro®/Patisiran	DSPC, Cho, DLin-MC3-DMA*, DMG-PEG2k	*i.v.*	TTR siRNA	TTR-mediated amyloidosis
Comirnaty®/BNT162b2	DSPC, Cho, ALC-0315*, ALC-0159	*i.m.*	mRNA	SARS-CoV-2
Moderna COVID-19 vaccine® /mRNA-1273	DSPC, Cho, SM-102*, DMG-PEG2k			

Abbreviations: ALC-0315, (4- hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis (2-hexyldecanoate), ALC-0159, 2-[(polyethylene glycol)-2000]-N,N-ditetra- decylacetamide. DLin-MC3-DMA, heptatriaconta-6,9,28,31-tetraen-19-yl-4- (dimethylamino) butanoate; DMPG, 1-α dimyristoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; PEG2k-DMG, polyethylene glycol 2000; SM-102, heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate. *i.m.* intramuscular, *i.v.* intravascular.
* ionizable lipid.

Translation of nanomedicines

Successful clinical translation of new medicines is a challenging task.[61] So far, there are many nanotechnology-based systems under clinical development, but very low success has been seen. A critical step for this process resides in the early stages of research. During these stages, careful selection of nanoparticles, within the infinite catalog of possible designs, is vital. Drug delivery systems with a robust design, stable, effective and non-toxic are essential to succeed and have a strong first preclinical data. Improvements in the initial stages of research will help in increasing the clinical success rate, for instance, more predictive models, targeted drug delivery technologies and a thorough understanding of the behavior and the route of nanoparticles in a living system.

In short, once formulated and after entering the body, intravenously injected nanoparticles have to overcome several barriers to reach the desired target. In the bloodstream, serum proteins and biomolecules interact and/or adsorb on the surface of the nanoparticles. This phenomena, known as 'protein corona' is a dynamic process impacting in the biological location and cellular uptake of the particles.[62, 63] Despite the efforts in the challenging task of protein corona analysis, the effect on nanoparticle composition and protein corona has not been fully elucidated. Several proteins are believed to help in the recognition of specific cellular receptors and eventually promote cellular internalization.[64, 65] For example, apolipoproteins such as apolipoprotein-E, that binds to the clinical formulation Onpattro®,[66, 67] facilitates the interaction with LDL receptors expressed on hepatocytes.

Besides interaction with proteins in the bloodstream, nanoparticle behavior is determined by their physicochemical properties. The chemical composition,[68] shape,[69, 70] size,[71, 72] stiffness,[73] hydrophobicity,[74, 75] and surface charge[76, 77] combined determine the biodistribution, cellular entry and circulation life time of nanoparticles. Precise control of these properties, by changing the particle composition, tuning the size, and/or exposing functional groups to the surface, allows one to tailor nanoparticle fate. It is important to note that study on the direct effect of an individual parameter in the biological identity of nanoparticles, in some cases, is challenging to assess *in vivo*. This is due to the complexity of the dynamic processes encountered by nanoparticles after systemic administration. In short, the material's chemical composition will define the nanoparticle surface properties, and thereby, corresponding interactions with different components in its surrounding. Shape is known to affect the curvature and surface contact with membranes, as well as phagocytosis by macrophages.[68] Nanoparticle size influences the renal clearance (limit 5,5 nm),[78] cellular internalization and engulfment of macrophages (>200 nm).[79] Diameter size of around 100 nm have shown prolonged circulation life-time and relatively low uptake by macrophages.[80] In fact, a 50 nm diameter is speculated as a recommended size since these particles favor kinetics and cellular uptake in comparison to particles of the same chemistry.[31, 72, 81] Stiffness influences circulation life-time, for example, softer nanogels circulate longer as to the stiffer counterpart, attributed to their capabilities of deformability.[73] Hydrophobicity is believed to improve cellular internalization of nanoparticles due to the increased enthalpic interaction with membranes.[74, 75] Likewise, cationic surface charge promotes endocytosis *via* interaction with negatively charged residues on the cellular surface membrane. Nevertheless, its use is limited by the associated cellular

toxicity.[82-84] This property leads to non-specific cellular internalization. In contrast, neutral (zwitterionic) surface charge, results in free circulation,[85] avoiding interactions and maximizing the exposure of nanoparticles to different tissues and cell types. Whereas negatively charged nanoparticles are characterized by a poor pharmacokinetic profile, preferentially found in liver and spleen, after rapid removal from circulation.[86]

Despite this flexibility in design, nanoparticles typically accumulate in non-desired tissues (also known as off-target) affecting the efficacy of the delivery system. This process is dominated by the reticuloendothelial system (RES), consisting of specialized cells: Kupffer cells (lining the sinusoids of the liver and the spleen) and fenestrated liver sinusoidal endothelial cells (LSECs). Due to the high endocytic capacity of these cells, RES main function is to maintain the body homeostasis by removing macromolecules from circulation and tissues, including pathogens (*e.g.* bacteria and viruses) and other foreign substances.[87-89] The ability to design nanomaterials that can selectively target or evade RES will contribute in the development of improved nano-therapies.

Clinical translation of nanomedicines has been primarily observed with lipid-based nanoparticles approach, however, many challenges remain.[90, 91] Although some general concepts about the influence of shape, size, surface charge on the fate of the nanoparticles are known, to which extend each of these properties affect the *in vivo* behavior? What are the optimal properties for a drug delivery system? How can we apply this knowledge to obtain the desired targeting? These are questions that are not fully understood. For these reasons and because of the poor predicting power of cell culture based-studies in nanomedicine, a model to screen multiple nano-systems is highly desired. Ideally, this model allows tracking of the particles *in vivo*, contributes to understand how the nanoparticle properties affect the *in vivo* fate, improves the design of delivery systems and potentially the success rate in the future medical applications.

1.2 Zebrafish as an animal model in the field of nanomedicine

The zebrafish *(Danio rerio)* is a teleost, non-mammalian vertebrate animal that originated from tropical freshwater. It was first used as a biological model by George Streisinger in the early 1980s,[92] since then the zebrafish has gained increased attention in research due to the wide range of advantageous features and is now one of the most studied animal models. The small size (from 0,7 µm in a single cell stage to 4 mm as larvae), high fecundity, ease of husbandry, relatively low cost of maintenance, external fertilization, and rapid development are only a few of the characteristics that attract researchers to select this animal for research studies.

As an experimental animal, zebrafish are extensively used in the area of developmental biology, regeneration, drug discovery, toxicity, cancer, inflammation, and molecular genetics. The comparison of fundamental and cellular events between zebrafish and mammals is supported by a known conservation in developmental and physiological processes among vertebrate species. For instance, lipid uptake/metabolism, and lipid transport are conserved and shows high similarity to humans.[93] Studies in this area can be performed on embryonic stages since most of the organs in the gastrointestinal tract are established at 2-4 days post-fertilization (dpf).[94, 95] The liver, however, is one of the organs that shows its full functionality about 120 hpf,[96] while the kidney shows glomerular filtration since 48 hpf a complete renal anatomy with glomerular and tubular filtration processing at 96 hpf.[97] Conservation at the genomic level was further confirmed in 2010s with the identification of 26206 protein-coding genes[98] and the sequence of the complete zebrafish genome.[99] These genes revealed the close relation of the zebrafish genome with that of mammals, having a 70% similarity with humans. Whereas 82% of human genes associated with diseases have a zebrafish orthologue.[99, 100] Genes associated with neurological disorders, cardiovascular disease, cancer, and vascular disorders have been identified and characterized in the zebrafish.[98, 99] In addition, comparative transcriptomic studies of different organs such as the heart,[101] liver,[102] lungs-swim bladder,[103] kidney,[104] or pancreas[105] between different species have demonstrated the good correlation of zebrafish analysis with higher organisms. Overall, this data support the functional conservation of genes and pathways between zebrafish and mammals and allow, to a certain extent, extrapolation of data obtained by zebrafish analysis.

Zebrafish key features for the development of efficient nanomedicines

Compared to other *in vivo* models, such as rodents, two particular characteristics make this organism suitable and attractive for research in nanotechnology: the optical transparency that offers the embryonic stages allows visualization and imaging, and the external fertilization facilitates the genetic manipulation (**Figure 2**). The combination of both characteristics provides a suitable strategy to shed light on the cellular mechanism involved in nanoparticle behavior.

The transparency of *Danio rerio* together with a variety of *in vivo* imaging techniques enables a non-invasive assessment of many biological processes through evaluation of the dynamics of fluorescent probes (*i.e.* proteins, synthetic dyes, quantum dots, *etc*.). The zebrafish development, for instance, has been tracked by microscopy and computational approaches resulting in a high-resolution map of embryogenesis in time.[106] Furthermore, incorporation of fluorescent dyes to nanoparticles allows the study of nanoparticle biodistribution after systemic administration by tracking their dynamics and fate in a whole living animal.[107] In this context, nanoparticles affinity for specific tissues can be visualized in detail providing information about cellular interactions involved in the underlying mechanism.

A range of tools are available to study nanomedicine biodistribution and nanoparticle-cell interaction zebrafish at the molecular level. This includes transgenic lines expressing fluorescent reporter proteins controlling promoter sequences for marked cell types,[108] mutants, antibodies, probes used to study mRNA expression of genes, *etc*. Established transgenic lines facilitate studies since it avoids non-specific labeling as a result of typical immunostaining of cells; such as *kdrl* and *mpeg1* lines expressing fluorescent proteins in endothelial cells and macrophages respectively. These two are of particular interest because nanoparticles, after parenteral administration, interact with: blood proteins in circulation, endothelial cells lining the vasculature, and blood resident macrophages. Tg(*kdrl:*eGFP)^{s843} [109] allows visualization of the vascular system and therefore quantification of nanomaterials in circulation over time (*e.g.* to determine pharmacokinetic values such as half-life), extravasation, and endocytosis by specific type of endothelial cells. Whereas the transgenic Tg(*mpeg1:*mCherry)^{gl23} [110] line label macrophages enabling the dynamic study of nanoparticles phagocytosed by macrophages. Other transgenic lines with possible applications in studying nanomedicine behavior are shown in **Table 3**.

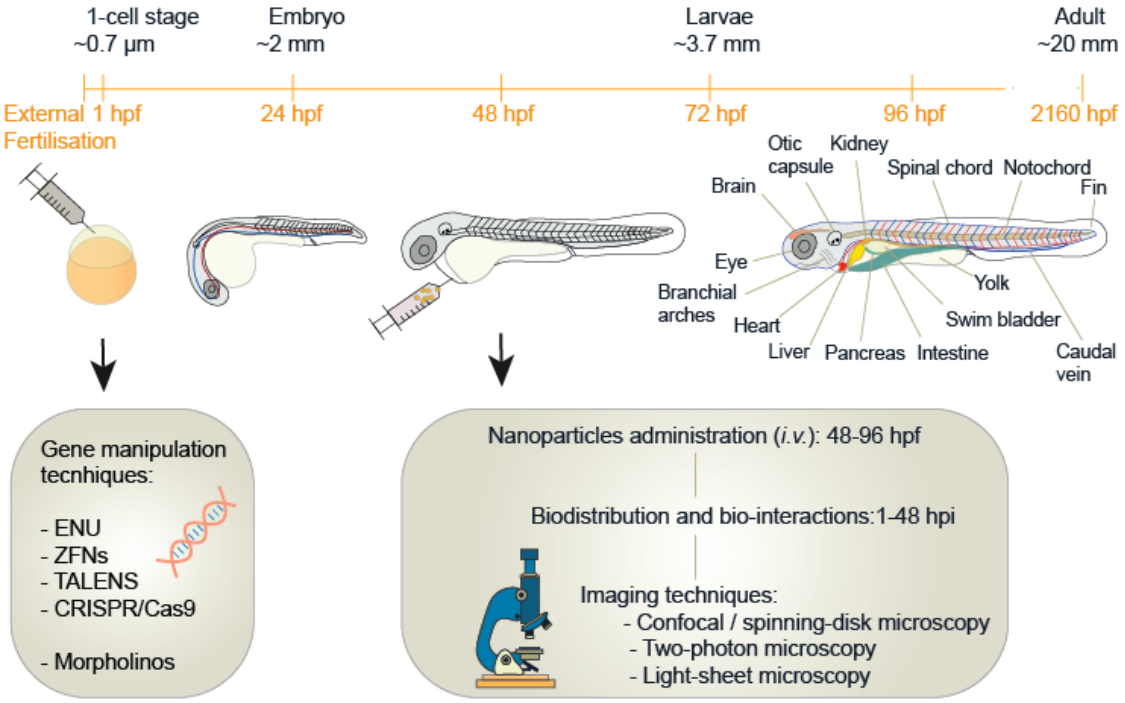

Figure 2. Development of the zebrafish and important features for nanomedicine. Timeline showing the zebrafish development from external fertilization (0 hours) up to 2160 hours post fertilization (hpf). Gene modification is performed at 0-0,75 hpf, in a 1-cell stage. Some of the techniques known for gene manipulation that have been applied in zebrafish to knockout genes are: ENU, ZFNs, TALENS and CRISPR/Cas9. Morpholinos are known to transiently knockdown genes of interest. At 24 hpf, the zebrafish embryo has a body length of about 2 mm. At this stage the circulatory system starts to develop. At 48 hpf, the embryo continue growing, circulatory systems is more develop, most of the organs are established. From this stage, fluorescently labeled nanoparticles can be *i.v.* administrated. Biodistribution of the nanoparticles can be tracked and bio-nano interactions can be studied with the help of different microscopy techniques such as confocal, spinning-disk confocal, two-photon, light-sheet. The main organs and other parts of the zebrafish are indicated in a larvae of about 96 hpf. The larvae continue growing until reaching the adult stage at 2160 hpf (3 months).

Furthermore, external fertilization of the zebrafish facilitates genetic manipulation. This characteristic provides the possibility to develop disease models and study the influence of a candidate gene in a disease state. In nanomedicine, this feature can also be applied to understand the mechanisms of nanoparticle-receptor interaction associated mediating uptake. The first attempt to modify a zebrafish gene was performed in the 90s employing chemical mutagenesis through a point mutation induced by *N*-ethylnitrosourea (ENU).[111] This methodology, based on forward genetics, carries a mutation for the next generation and requires large screenings to determine phenotypic changes to be

further studied. Later, genetics evolved to more developed reverse approaches enabling random (*Tol2* transposons)[112] and targeted insertions of exogenous DNA into the genome. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS) have also been used to mutate specific genes in the zebrafish.[113, 114] More recently, CRISPR/Cas9 appeared as an efficient gene-editing technique due to its ability to induce double-strand breaks within an identified genomic location (similar to ZFNs and TALENS) and its potential for targeting multiple genomic regions.[95, 115-117] Co-injection of single guide RNA (sgRNA) -including a 20 nucleotides target sequence- along with Cas9 mRNA in a single cell stage of a zebrafish zygote could result in a mutant carrier. After identifying the founder carrying the mutation and some crosses for new generations, a homozygous mutant may be available for further studies.

Table 3. Selected fluorescent transgenic lines of interest for the evaluation of nanomedicines.

Transgenic line	Marker	Application in nanomedicine
*kdrl:*eGFP [109], *kdrl:*mCherry, *kdrl:*mTurquoise *fli-1:*eGFP, [118] *gata1:*dsRed [119]	Endothelial cells (vascular system)	▪ Nanoparticles in circulation/ circulation life time of nanoparticles ▪ Extravasation of nanoparticles ▪ Endocytosis of nanoparticles by endothelial cells
*mpeg1:*mCherry, *mpeg1:*GFP [110]	Macrophages	▪ Sequestration of nanoparticles by macrophages ▪ Targeting of macrophages
*cldn5a:*GFP [120]	BBB and choroid plexus	▪ Targeting of brain endothelial cells ▪ Extravasation
l-fabp: GFP [121]	Hepatocytes	▪ Uptake of nanoparticles by liver
*live1:*dsRed [122]	Lymphatic endothelial cells	▪ Lymphatic uptake
*wt1b:*GFP [123]	Pronephros	▪ Kidney uptake ▪ Blood filtration of nanoparticles

Abbreviations: BBB, blood brain barrier; SECs, sinusoidal endothelial cells.

Site-specific modifications are identified by genotyping (sequencing or fluorescent PCR) followed by phenotypic analysis. In addition, transient knockdown of a targeted gene with morpholinos is also applied in zebrafish.[124] This strategy relies on the use of synthetic oligo, antisense morpholino, that inhibits the translation of a complementary mRNA by preventing initiation or splicing. Although it is a fast and low cost, high inconsistencies (up to 80%)[125] in phenotypes due to off-targeting inhibition in the use of morpholinos compared to a knockout made its efficacy questionable in respect to false positives.

The advantages of the zebrafish in nanotechnology are evident, useful and in principle provide a great addition compared to other models. The tools available in zebrafish research and advances in new technologies in other related fields, such as imaging and genetics, could offer constant improvement and increase its potential.

State-of-the-art of the zebrafish in the study of nanoparticles

While it is evident that the zebrafish share many cellular and physiological mechanisms with mammals and that they possess attractive characteristics to be preferred as a model in many research areas. It was not until very recently that this model started to be used to study nanomedicines.[126-128] A search in Pubmed reveals the limited amount of publications per year in the last decade indicating that despite the already highlighted zebrafish features, the model is relatively new and has not been deeply explored. Compared to the total amount of publications in zebrafish in 2019, 3,8% of the publications included nanoparticles research and less than 1% represented studies in nanomedicine. Such a difference can be explained by the application of the zebrafish in the toxicological analysis of nanoparticles. In addition, an important contribution of this organism in the study of cancer (already reviewed in references [126, 129, 130] and therefore not discussed in this thesis), gene therapy, and phagocytic by macrophages have been reported.

As a model in toxicology, zebrafish is useful due to their sensitivity to chemical exposure during early stages and the amenability to monitor phenotypical changes. Nanoparticle toxicology studies in the zebrafish, as a relevant alternative to rodents, has been recently reviewed.[131-133] It relies mainly on the toxicity assessment after direct exposure of the zebrafish to water containing nanoparticles.[132] This allows the study of molecular mechanism, functional changes and transcriptional gene alterations.[134, 135] Markers associated with intestinal inflammatory responses, for example, were used to study the toxicity

of metal and polystyrene nanoparticles.[135] This type of administration, however, should consider variability in the absorption, dosages, as well as stability of the nanoparticles. Therefore, toxicology experiments after systemic administration are also recommended.

In the study of gene therapy mediated by non-viral delivery systems,[136] zebrafish embryos, combined with fluorescent materials (DNA, mRNA, small interfering siRNA), enables visualization of genetic material delivered by nanoparticles and functional activity. Not only gene silencing[137, 138] has been demonstrated with zebrafish embryos but also mRNA transcribing fluorescent proteins. A pilot study using a non-delivery system, LNPs, as nanocarriers showed delivery of genetic encapsulated mRNA, and fluorescent expression (eGFP) as a delivery readout.[139] Important research efforts have been the focus in the development of new LNPs,[140, 141] the potential of the zebrafish to assess these nano-sized technologies and to further understand the interactions between the vector and their host could pave the way to better translations of gene therapies in higher organisms. With the help of fluorescent genetic material (DNA, small interfering siRNA,) the transfer and expression of exogenous genes in specific cell-type can be investigated in zebrafish. In this context, silencing of specific genes by siRNA-mediated delivery in the study of cardiac diseases seems to be the more explored area within gene therapy in adult zebrafish. For instance, polymer and dendrimer-based nanoparticles showed functional siRNA delivery by mediating silencing of specific genes in the heart.[142, 143] In addition to the functional assessment, more recently the distribution of polyamidoamine dendrimer-based nanoparticles in endothelial cells, cardiomyocytes, leucocytes, and macrophages were quantified.[144]

In recent years, *Danio rerio* has gained attention as an attractive research model. Surprisingly, the relatively low amount of studies in the field of nanomedicine reflects that, in this context, it is a model that remains rather unexplored. Although high conservation between zebrafish and mammals is known, a potential challenge in terms of translation due to differences in specific mechanisms, gene duplication, absence of proteins, differences in development, can be foreseen and need to be taken into consideration.

1.3. Scope and motivation of the thesis

Nanoparticles composed of lipids have shown to improve the therapeutic effect of various drugs; however, there is clearly room for improvement. This thesis aims to improve the mechanistic understanding and the fundamental *in vivo* behavior of nanostructures after *i.v.* administration. This is achieved by applying the zebrafish as a model organism to study fundamental aspects of nanoparticles distribution and provides the possibility to identify cellular interactions and mechanism of clearance *in vivo.* Motivation relies mainly on the potential that nanoparticles have as a delivery vehicle and the lack of *in vivo* information about the bio-nano interaction involved. Therefore, this thesis starts with the basics, establishing a zebrafish model, assessing the influence of surface properties on the behavior of nanoparticles *in vivo* and identifying key biological interactions with liver cell types in a molecular level (**Chapter 2-3**). Next, the scope is changed to design nano-systems that target specific cell types (**Chapter 4-6**).

After an introduction to the field of nanomedicine and zebrafish as a general *in vivo* model in **Chapter 1**, we established the zebrafish as a model to study nanoparticles in **Chapter 2** and showed that fluorescent nanoparticle biodistribution can be tracked and visualized in embryonic zebrafish. In addition, by applying the gene-editing technique CRISPR/Cas9 to zebrafish, **Chapter 2** revealed the identification of Stabilin-2, an important scavenger receptor that recognizes and removes macromolecules and a range of nanoparticles from circulation. Stabilin-2 is expressed in a subset of endothelial cells in the zebrafish, named as scavenging endothelial cells, functionally homolog to LSECs in mammals. However, other receptor, were also involved in the clearance of anionic nanoparticles by the liver, since part of particles studied were not influenced by Stabilin-2. In **Chapter 3**, we continued investigating the clearance of these nanoparticles by studying additional scavenger receptors expressed in LSECs. Here, the generation and characterization of a single Stabilin-1 and a double Stabilin zebrafish mutant lead to unravel the role of Stabilin-1 in the uptake of nanoparticles. Stabilin-1 was identified as responsible for the clearance and uptake of small anionic nanoparticles (6-30 nm). In addition, we revealed that together with Stabilin-2, Stabilin-1 contributes in the clearance of bacterial lipopolysaccharide from circulation and anionic nanoparticles of about 100 nm size.

Preceded by an understanding of the fundamental behavior of nanoparticles, this thesis changed the scope to investigate nanomedicine able to target specific cell

types or with control over nanocarriers-cell interaction. To date, several strategies have been employed to design nano-systems capable of delivering cargo triggered by an external stimulus. These systems offer a better spatiotemporal control for delivery of the cargo. In **Chapter 4**, using light as an external trigger, we developed liposomes capable of switching the surface charge *in situ* and *in vivo*, changing from a near-neutral circulating liposomes to a cationic formulation, resulting in the intracellular uptake and delivery of an impermeable cargo. Switching of the surface charge occurs due to the incorporation of a photoactive lipid into the liposomes, as one of the two components of this simple formulation. Simultaneous interactions of the liposomes with endothelial cells and macrophages were shown to be dependent on the transition of the surface charge. This behavior, as well as endocytosis and payload delivery was visualized in the zebrafish embryo in real time.

In **Chapter 5**, a serendipitously found liposomal formulation is studied and characterized. This PAP3 formulation consist of two lipids (DSPC and DOaG) and, to our surprise, accumulate in brain endothelial cells in the zebrafish. Physicochemical characterization of these liposomes, including cryo-transmission electron microscopy (CryoTEM), revealed an uncommon phase-separated 'parachute' morphology, which correlates with the specific targeting *in vivo.* Structurally, the DOaG lipid resemble diacylglycerol (DAG, known to perturb lamellar membranes structure) and in combination with DSPC, form a hydrophobic 'bleb' as a result of a phase separation. Mechanistically, PAP3 liposomes in zebrafish hijack an endogenous triglyceride lipase-mediated pathway of plasma lipid transport and metabolism to selectively target, and be taken up by, endothelial cells *in vivo* which express endothelial lipase (*LIPG*) in embryonic stages. In mice, liposome predominantly accumulate within the liver and spleen, matching with the high expression of endothelial lipase within these metabolic organs.

Using the concepts learned in previous studies on how the liposomes distribute according to their physicochemical properties, we describe in **Chapter 6** a rationally designed LNP formulation able to preferentially target hepatic RES. Here, mRNA was preferentially delivered using an anionic LNP (srLNP) formulation. Biodistribution of srLNPs and cytoplasmic delivery of the mRNA was successfully shown in zebrafish and we demonstrated the selective expression of a fluorescent protein in hepatic cell types at cellular resolution. Mechanistically, we revealed that targeting of SECs is mediated by scavenger receptors Stabilin-1 and Stabilin-2. Furthermore, translation in mice confirmed the srLNP biodistribution, uptake, cytosolic delivery and protein expression of

hepatic RES mice cells. This proves the importance of understanding the mechanism of the designed delivery systems and the ability of the zebrafish model to predict and optimize nanoparticle formulations.

In **Chapter 7** a step-by-step description of a protocol used throughout this thesis is provided. This ensures reproducibility of the experiments performed, such as intravenous injection, imaging and analysis of nanoparticles in the zebrafish.

In **Chapter 8,** the obtained results are discussed in general terms, in the context of nanomedicine, and includes concluding remarks.

1.4. Abbreviations

ALC-0315	(4- hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis (2-hexyldecanoate)
ALC-0159	2-[(polyethylene glycol)-2000]-N,N-ditetra-decylacetamide
BBB	blood brain barrier
Cho	cholesterol
CRISPR/Cas9	clustered regularly interspaced short palindromic repeat/Cas9
CryoTEM	Cryo-transmission electron microscopy
CV	caudal vein
DAG	diacylglycerol
DLin-MC3-DMA	amino lipid dilinoleylmethyl-4-dimethylaminobutyrate
DMPC /G	1-α-dimyristoylphosphatidylcholine / glycerol
dpf	day(s) post fertilization
DOPC /S	1,2-dioleoyl-*sn*-glycero-3-phosphocholine / serine
DSPC /G	1,2-distearyl-*sn*-glycero-3-phosphocholine / glycerol
DSPE	1,2-distearyl-*sn*-glycero-3-phosphatidylethanolamine
ENU	*N*-ethylnitrosourea
EPC/G	egg phosphatidylcholine/glycerol
hpi	hour(s) post injection
HSPC	hydrogenated soy phosphatidylcholine
i.m./ i.v.	intramuscularly / intravenously
LDL	low density lipoproteins
LNP	lipid nanoparticle
LSECs	liver sinusoidal endothelial cells
PEG	polyethylene glycol
PLGA	poly(lactic-co-glycolic)
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
RES	reticuloendothelial system
SEC	scavenger endothelial cells
sgRNA/siRNA	single guide RNA / small interfering RNA
SM-102	heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6 (undecyloxy)hexyl amino)octanoate
TALENS	transcription activator-like effector nucleases
TTR	transthyretin
VLDL	very low density lipoproteins
ZFNs	zinc finger nuclease

1.5 References

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