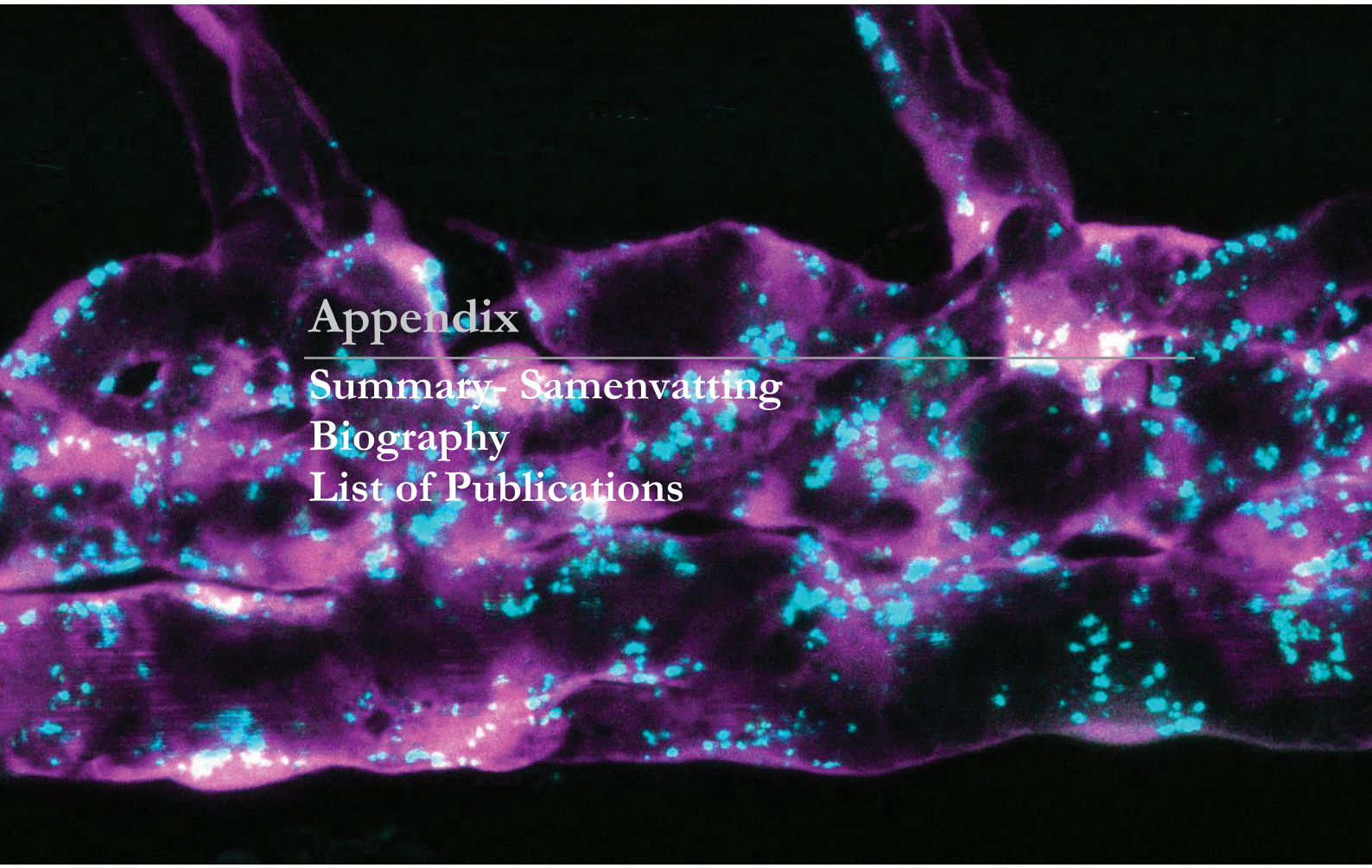
Towards a mechanistic understanding of nanoparticle behavior using zebrafish

Arias Alpizar, G.



Appendix

Summary- Samenvatting
Biography
List of Publications

Image: Venous endothelium (magenta) after intravenous injection of fluorescently labelled nanoparticles (cyan) in Tg(*lyve1*:DsRed) zebrafish embryo.

Summary

Clinical translation of efficient and targeted drug delivery nanosystems is challenging, partially due to an insufficient knowledge of the mechanisms influencing nanoparticle circulation, uptake and the identification of molecular components in these mechanisms. Due to the dynamic nature of the involved mechanisms, an *in vivo* assessment is strictly required. The work described in this dissertation contributes to the mechanistic understanding of the behavior of primarily lipid-based nanoparticles *in vivo*. To achieve that goal, we used the zebrafish as an *in vivo* pre-screening model of nanoparticles, by studying the biodistribution and to unravel specific interactions at the cellular level. As the main strategy, fluorescently labeled nanoparticles were injected in zebrafish embryos and imaged with confocal microscopy to track the intravenously administered nanoparticles. This approach enables the investigation of the fundamental behavior of nanoparticles, correlation of the physicochemical properties of the formulated lipid-based nanoparticles with their biodistribution and identification of important nano-bio interactions. Zebrafish established transgenic lines expressing fluorescent proteins in specific cell-types were used to study specific interactions. In addition, genetically modified zebrafish applying CRISPR/Cas9 were generated. These strategies not only show key mechanistic features of nanoparticles in circulation, but also promote the rational design of more efficient nanoparticles systems, able to preferentially target specific cell-types *in vivo*.

Chapter 1 introduces the field of nanomedicine, describes the design and properties of the lipid-based nanoparticles and provides a list of the clinically approved intravenously (*i.v.*) administered lipid-based drug delivery systems. This overview revealed the necessity of *in vivo* models to support the selection of appropriate delivery nanotechnologies and improve the success rate in the clinical translation of nanomedicines. Comprehension of the nanoparticle journey and associated nano-bio interactions to reach the desired target is required to achieve this goal. In this chapter, the physiological barriers encountered by nanoparticles in circulation (interaction with immune cells, clearance of particles, *etc.*) are described. In addition, the zebrafish model is proposed to visualize and investigate the nanoparticle journey *in vivo*. **Chapter 1** highlights key features of the zebrafish, such as optical transparency and ease of genetic manipulation, making them an attractive model for the development of more efficient nanomedicines.

In **Chapter 2**, the embryonic zebrafish is established as a model to study the *in vivo* behavior of nanoparticles. The combination of the transparency of this organism

with fluorescent labeled nanocarriers and visualization by confocal microscopy revealed fundamental aspects in the behavior of a wide range of nanoparticles used. Here, biodistribution studies and identification of cellular interactions with nanoparticles were analyzed in the caudal region of the zebrafish. Importantly, this region displays a subset of endothelial cells that are in close homology to liver sinusoidal endothelium in mammals. This was confirmed by the conserved uptake of oxidized LDL, lithium carmine, DOPG liposomes and fluorescent hyaluronic acid allowing translation to mice. In addition, **Chapter 2** revealed the identification of Stabilin-2 receptor in the uptake of primarily anionic nanoparticles. Stabilin-2 is a scavenger receptor that recognizes and removes biomolecules, bacteria, and other ligands from circulation. As shown in this chapter, the Stabilin-2-nanoparticle interaction is of particular importance to nanomedicine delivery since it can be either blocked by inhibitors (*i.e.* dextran sulfate) increasing the circulation life time of nanoparticles or exploited to target a specific liver cell-type (*e.g.* liver sinusoidal endothelial cells, LSECs).

In **Chapter 3**, other scavenger receptors abundantly expressed in mice liver endothelium and in the caudal vein region of the zebrafish embryo were investigated to study the clearance of anionic nanoparticles. CRISPR/Cas and *in situ* hybridization methodologies were applied to successfully generate and characterize a single *stabilin-1* mutant and a double *stabilin-1* and *stabilin-2* zebrafish mutant. The role of Stabilin-1 receptor on the uptake of nanoparticles was studied, revealing that nanoparticle size is one of the key parameters determining the differential uptake between the two Stabilin receptors. Stabilin-1 was identified as responsible for the clearance and uptake of small anionic nanoparticles (6-30 nm). In addition, this investigation revealed that both Stabilin receptors contribute to the clearance of anionic nanoparticles with diameter of ~100 nm. Finally, we identified an endogenous Stabilin ligand, the endotoxin lipopolysaccharide, responsible for toxicity and immune activation in mammals. This ligand was found to be cleared from host circulation by Stabilin-1 and Stabilin-2.

Preceded by an understanding of the influence of the surface charge in the behavior of nanoparticles, the research in **Chapter 4** focuses on the design of a nano-system capable of delivering a cargo triggered by an external stimulus. Generally, this approach is attractive because it offers a better spatiotemporal control for delivery of the cargo. Here, liposomes capable of switching the surface charge *in situ* and *in vivo* using light as an external trigger were developed. Liposomes were composed of a neutral lipid facilitating circulation and a photoactive lipid that, upon stimulus, switches the surface charge from near-

neutral to cationic. This change in the surface properties results in endocytosis and delivery of the liposomal content. After formulation and biophysical characterization, the liposomes were intravenously administered in zebrafish. Liposomes freely circulated allowing access to any vascularized tissue. Upon exposure to UV light, the liposomes adhered rapidly to all endothelial cells followed by internalization. This demonstrated stimulus-controlled endocytosis and payload delivery. Furthermore, dynamic and simultaneous interactions of the liposomes with endothelial cells and macrophages were found during the transition of the surface charge.

Chapter 5 a serendipitously found liposomal formulation targeting the brain vasculature of the zebrafish was studied. Here, cryo transmission electron microscopy (CryoTEM) analysis and *in vivo* biodistribution studies were combined to understand the observed tissue specificity. Liposomes, named PAP3, composed of two lipids (DSPC and DOaG) showed accumulation in brain endothelial cells (bECs) in the zebrafish. Biophysical characterization of PAP3 liposomes with CryoTEM revealed an uncommon 'parachute' morphology. This can be attributed to the observed phase separation between DOaG and DSPC lipids. Furthermore, the presence of phase separation domains in the liposomes was required to obtain the specific targeting in the zebrafish larvae. Mechanistically, it was also demonstrated that the recognition and binding of PAP3 liposomes are inhibited by heparin and decreased by the endothelial lipase inhibitor XEN445. This data suggests that PAP3 liposomes hijack an endogenous triglyceride lipase-mediated pathway of plasma lipid transport and metabolism, targeting endothelial cells expressing endothelial lipase (*LIPG*), highly expressed in bECs in zebrafish embryo. In mice, liposomes predominantly accumulated within the liver and spleen, matching with the high expression of endothelial lipase within these metabolic organs and opening the opportunity to further study this behavior in mice.

In **Chapter 6,** a rationally designed lipid nanoparticle (LNP) formulation containing mRNA able to preferentially target the hepatic reticuloendothelial system (RES) was developed. To design srLNPs, a single zwitterionic lipid (DSPC) within the lipid composition of the clinically approved Onpattro®, was replaced with DSPG. This replacement changes the surface charge of the LNPs, from neutral to anionic, resulting in redirecting preferentially targeting to the hepatic RES. The *in vivo* behavior of LNP formulations were compared showing that mRNA was preferentially delivered using srLNP formulation intravenously administrated in the zebrafish embryo. Biodistribution of srLNPs and cytoplasmic delivery of the mRNA in zebrafish demonstrated the selective expression of a fluorescent protein in hepatic cell types at cellular resolution. This study revealed that srLNPs

effectively target scavenging endothelial cells (SECs) mediated by scavenger receptors Stabilin-1 and Stabilin-2. This was confirmed with the help of our previously established *stabilin* double knockout (described in Chapter 3). Furthermore, validation in mice confirmed the srLNP biodistribution, uptake, cytosolic delivery and protein expression of hepatic RES mice cells, opening up opportunities to treat diseases associated with RES.

Chapter 7 provides a step-by-step protocol for the intravenous administration of nanoparticles in the zebrafish embryo, a technique used throughout the studies described in this thesis. This chapter highlights the importance, cost effectivity and versatility of the zebrafish to study nanomedicine, provides a list of the materials and equipment required to ensure reproducibility of a range of experiments. This includes liposome formulation, detailed description of how to perform intravenous injection in a zebrafish embryo, application of UV-light to trigger the release of photocages in the zebrafish embryo, imaging and analysis of nanoparticles in primarily the caudal vein region of the zebrafish.

The last chapter, **Chapter 8**, combines all studies presented in this dissertation in a general discussion resulting in some concluding remarks.