Towards a mechanistic understanding of nanoparticle behavior using zebrafish

Arias Alpizar, G.

Chapter 8

General Discussion & Concluding Remarks

Image: Tissue resident macrophage (in magenta) expressing eGFP (in yellow) after delivery of mRNA with srLNPs (cyan) looking at a non-transfected macrophage.

8.1 General discussion

Despite remarkable advances in nanomedicine, (*i.e.* increasing the therapeutic index of delivered carriers, protecting cargos), this field has been full of expectations with relatively slow progress, hampered by underestimated challenges (*i.e.* clinical translation). This situation is reflected in the limited amount of nanoparticles that reached the final approval to be used as therapeutics. Over the past decades, most studies have been mainly concentrated in the design of new (and sometimes very complex) systems to achieve targeted drug delivery, often disregarding the fundamental and mechanistic behavior of nanoparticles *in vivo* and the corresponding biological interactions in health and diseases states.

Conventional *in vivo vs. in vitro* nanoparticle assessment

In vitro and *in vivo* studies have been traditionally proposed to assess the effect of nanoparticles in biological systems. Nowadays, *in vitro* technologies that aim to simulate the physiological environment (*i.e.* organ-on-a-chip, extracellular matrix) are emerging. However, *in vitro* tests relying on the exposure of nanoparticles to culture cells is still frequently used. This strategy favors the rapid test of many formulations but unfortunately, prediction of the *in vivo* behavior of nanoparticles and translation of data obtained only by *in vitro* studies, is often unsuccessful.

Static conditions of *in vitro* studies do not consider dynamic key processes affecting the fate of nanoparticles, for instance blood-proteins adsorbed on the nanoparticle surface, or the sequestration and clearance of nanoparticles by immune (*i.e.* macrophages) and other cell types. In addition, the rapid dedifferentiation of particular cells in culture, such as LSECs,[1] not only limit long-term culture but also changes the profile and functionality *in vitro,* affecting receptor-nanoparticle interactions*.* For these reasons and considering the diversity of nanoparticle systems, it becomes clear that in depth analysis of nanoparticle-bio interactions strictly requires *in vivo* experiments. To date, rodents are the conventional and required biological system used to test stability in blood, circulation life-time and delivery efficiency of their cargos. Frequently nanoparticles tested in rodents are unable to deliver (therapeutic relevant) drugs to the desired tissue, often without a proper understanding of the reason(s) for the failure. This reveals the challenging task of translating nanomedicines to the clinic and the need for additional model(s) that could allow

nanoparticle pre-screening to obtain improved nanomedicine formulations and contribute in the comprehensive recognition of involved biological interactions.

Zebrafish as an accurate model in nanomedicine research

In this thesis, the accuracy and efficacy of the zebrafish embryo in nanomedicine is demonstrated and applied to design new strategies for preferential targeting. The zebrafish represents a suitable alternative, to bridge the knowledge gap between *in vitro* and *in vivo* studies, by investigating the complex journey of nanocarriers and their potential interactions after systemic administration. Therefore, an useful addition to the design of improved drug delivery systems.

The practicality and the unique advantage of optical transparency of the zebrafish enable imaging techniques to track fluorescently labeled nanoparticles in real time, thereby, the screening of big nanoparticle libraries. These characteristics not only offer the opportunity to correlate the physicochemical properties of designed nanoparticles (and variations) to the behavior in an multiorgan system, but also gives the possibility to discover novel (unexpected) nanomedicines. Furthermore, most of the biological processes controlling the uptake of nanoparticles can be resolved in the zebrafish due to the overall conservation at a cellular level of physiological processes involved. Besides the fundamental behavior of engineered nanoparticles, processes that can be studied in this model include: the correlation between physicochemical properties and cellular responses, pharmacokinetics, biodistribution of (functionalized) nanoparticles, dynamic processes in real time such as endocytic routes, phagocytosis by macrophages, delivery of payloads (*i.e.* small molecules, genetic material), mechanisms of clearance, and preferential targeting. From the functional point of view, the ease to generate mutants or transgenic lines, by using CRISPR/Cas9 or *Tol2* respectively, facilitates the study of molecular interactions with cell-types and/or receptors by comparing the interaction of nanoparticles in the absence or presence of the functional protein of interest.

Additional innovative techniques can be exploited in combination with the zebrafish model used in this thesis, especially in the field of genetics and microscopy, which are constantly evolving and introducing breakthroughs. For example, more detailed transcriptomics data that allows information about the zebrafish gene expression at different stages, from 1 to 5 dpf, in a transcriptomics atlas[2] will contribute to the improvement of the zebrafish in the nanomedicine field once a specific receptor is targeted. Additionally, advancements in microscopy techniques applied to a zebrafish, such as improvements in the

tracking of (single) nanoparticles with high-resolution images, or the combination of serial sectioning cryo-preserved zebrafish tissue with scanning electron microscopy that determines the cellular fate of electron-dense particles[3], will directly benefit and make a more powerful zebrafish model for developing better nanomedicines.

It would be, however, wrong to pretend that the zebrafish replaces rodent experiments. Instead, as an *in vivo* pre-screening tool of nanomedicines, zebrafish is intended to be used as a model to optimize the infinite amount of nanomedicine platforms and mechanistically understand fundamental interactions. Importantly, differences between embryonic stages, compared to adults, or between organisms (*e.g.* ratio between cell types, genetic expression) should be considered. Therefore, validation with rodents once a nanomedicine formulation is optimized, is still required. In this way, the use of additional adult animals or mammal studies could be significantly decreased, supporting the ethical guidelines of the 3 R's (replacement, refinement, reduction) in the use of experimental animals. Furthermore, long term (>5 dpf) zebrafish experiments for the study of adaptive immune responses (*i.e.* in the development of vaccines) require, as mice, ethical approval of the regulatory animal experiment agencies.

Key biological mechanisms underpinning nanoparticle behavior and applications

The biological mechanisms investigated throughout this thesis includes the effect of physicochemical properties (*e.g.* surface charge, size, chemistry) on the biodistribution of nanoparticles, endothelial cell uptake, and clearance by the hepatic RES (*e.g.* LSECs and KCs). Especial attention was dedicated to anionic nanoparticles and their uptake mediated by interaction with scavenging receptors Stabilin-1 and Stabilin-2, described in **Chapters 2** and **3**.[4, 5] This process is of vital importance, because it represents one of the main hepatic clearance routes of nanoparticles after systemic administration, for exogenous nanoparticles (*e.g.* polymeric, inorganic, virus-like). **Chapter 2** and **3** provides a relevant model to study the effect of these two scavenging proteins by generating the *stabilin 1- stabilin 2* double knockout zebrafish with CRISPR/Cas9. To note, double knockout experiments in mice are limited due to the low viability as a result of a kidney failure.[6] Here, it was shown that prevention in the clearance of LSECs enhances the pharmacokinetics of anionic nanoparticles; whereas targeting of LSECs allows the development of nanotherapies of liver-related diseases.

Convinced that a proper understanding of mechanisms related with uptake and clearance of nanoparticles represents a solid foundation to design simple and effective nanosystems, we implemented the fundamental knowledge acquired during the previous studies, described in the first part of the thesis, to rationally design two new liposomal formulations with targeting specificity or control over cell uptake. In **Chapter 4**, to control the cargo delivery with an external stimulus, long circulating liposomes composed of two lipids were studied.[7] Upon applying a light trigger, the surface charge changed, from near-neutral to cationic, and internalization of endothelial cells was observed with concomitant delivery of the cargo. This study revealed interesting mechanistic aspects associated with the rapid switching of the surface charge in real time *in vivo*, the behavior of cationic liposomes (*i.e.* interactions with macrophages and endothelial cells) and the importance of selecting a correct light dose to induce efficient photolysis and, thereby, a more efficient and controlled drug delivery system. Clinical relevance of light-based technologies have been proven and reviewed.[8] In this proof of concept study UV-light, which has a limited penetration depth, was used. However, longer wavelength sensitive photocleavable lipids can be used to circumvent this limitation.

A screening of nanoparticles formulated with a library of synthetic lipids resulted in the serendipitously discovery of a novel formulation able to target brain endothelial cells in zebrafish larvae. These liposomes are composed of only two lipids, with no additional targeting functionality and characterized by a phase-separated morphology as revealed by CryoTEM. By systematically varying the lipid composition, it was shown that a protrusion is required for selective liposome accumulation in the zebrafish. Moreover, the challenge of understanding the mechanism led us to compare the liposome fate with endogenous lipid transport. The involvement of a triglyceride lipase mechanism was shown and emphasizes the importance to look into endogenous lipid transport processes to comprehend mechanisms related with the uptake of lipid-based nanoparticles. Validation in rodents, injected with a radiolabeled formulation to allow PET imaging, supported our findings in wildtype mice; however, further studies will be required to further investigate the mechanism. This preferential targeting of endothelial cells via triglyceride lipase mechanism is described in **Chapter 5.**

Recently, mRNA-based therapies used LNPs as a carrier were designed and approved to treat the world-wide COVID-19 pandemic caused by SARS-CoV-2 (**see Chapter 8.2**). In **Chapter 6**, a simple lipid change in the already clinical approved Onpattro® LNP formulation results in the preferential targeting of

hepatic RES in mice. The rationale behind the lipid change was based on the knowledge acquired to preferentially target LSEC *via* Stabilin-1 and Stabilin-2 receptors using anionic nanoparticles. Biodistribution and cellular interactions of these two formulations were first studied in the zebrafish, including the previously generated *stabilin* double knockout to confirm the mechanism. Finally, the key findings were validated in mice demonstrating successful translation and the challenging task of preferentially target delivery.

In general, the findings in this thesis contribute in the comprehension of nano delivery systems *in vivo*. Liver-associated diseases (*e.g.* hepatic RES or EL as key players) are candidates for possible applications of nanoparticles, administered intravenously, described in this work. The target of other tissues or cell types than the liver becomes more challenging, due to off-targeting effects of particles accumulated in organs involved in clearance. Besides their applications, it is important to highlight the tendency to redirect the research in nanomedicine to a more rational design mediated by the understanding of the biological mechanisms occurring *in vivo*. This, undoubtedly, strengthens the knowledge needed to further apply it to the improvement of, not only new, but also existing therapies.

8.2 Concluding remarks

Deliver a cargo to the right place in a living system is not a simple job and requires a thorough understanding of the complete route and interactions that could hamper/help in the main task. The identification, in the early stages of research, of biological interactions with nanoparticles, at cellular resolution and in real time, and the comprehensive *in vivo* behavior provide opportunities to improve existing and new nanotherapies.

The investigation in this thesis has contributed to a better understanding of the fundamental and mechanistic behavior of the nanoparticles *in vivo* by using the zebrafish as a highly predictive and convenient model. Furthermore, most of the key findings were validated in rodents, indicating its suitability and predictability when translation is required. Many challenges remain though, novel molecular interactions to be discovered such as the role of other scavenger receptors and macrophages in the process of nanoparticles, understanding of the exact role of specific proteins adsorbed on the internalization of nanoparticles, identification of proteins that induce nanoparticle selective targeting (*e.g.* tumors, specific cell-types -especially outside of the liver-), effective strategies to

prevent/target macrophage uptake and the manipulation of nanoparticle interactions with immune cells to trigger immune responses.

Recently, after years of study, the approval of the first nanoparticle-based gene therapy drug in the clinic acknowledges the significant potential of nanotechnology. This new drug paved the way to a new era where nanomedicine vaccines were developed rapidly as a strategy to control a global pandemic situation. Nanomedicine platforms delivering a mRNA genetic sequence encoding for the stabilized viral spike (S) protein to the host cells with LNP as a carrier have demonstrated, so far, an innovative, efficient and accurate prophylactic strategy against the SARS-CoV-2 virus. Delivered mRNA antigenic proteins into the cytosol enables the antibody production that strengthens the immunological system to attack the virus if required. LNPs represents the most suited example of the new direction that nanomedicine is experiencing. This unique current situation, were two LNP-mRNA vaccines[9, 10] are approved by the World Health Organization and other nanotechnologies[11] show the ability of nanomedicine to design targeted drug delivery technologies. Likewise, it opens opportunities in the development of other vaccines, that are being developed, to treat lethal virus-caused diseases (*i.e.* Ebola, seasonal flu, cytomegalovirus), rated with high mortality worldwide and it indicates a shift of the nanotechnology towards a more advanced biotechnology application to provide personalized medicine such as protein replacement, gene editing applications specific sequences of target disease-causing genes, and cancer nanovaccines. Overall, in this new era of nanomedicine, zebrafish represents a robust model in the pre-screening and optimization of LNPs, as demonstrated in this thesis.

Finally, advances in parallel research fields like genetics, epigenetics, imaging techniques in combination with *in vivo* models as research tools, such as the zebrafish presented in this thesis, reinforce strategies to continue the fascinating research of molecular interactions affecting the behavior and uptake of NPs and that will contribute to the improvement of nano-therapies.

8.3 References

1. Geraud, C.; Schledzewski, K.; Demory, A.; Klein, D.; Kaus, M.; Peyre, F.; Sticht, C.; Evdokimov, K.; Lu, S.; Schmieder, A.; Goerdt, S., Liver sinusoidal endothelium: a microenvironment-dependent differentiation program in rat including the novel junctional protein liver endothelial differentiation-associated protein-1. *Hepatology* **2010,** *52* (1), 313-326.
2. Farnsworth, D. R.; Saunders, L. M.; Miller, A. C., A single-cell transcriptome atlas for zebrafish development. *Dev Biol* **2020,** *459* (2), 100-108.
3. Vidavsky, N.; Akiva, A.; Kaplan-Ashiri, I.; Rechav, K.; Addadi, L.; Weiner, S.; Schertel, A., Cryo-FIB-SEM serial milling and block face imaging: Large volume structural analysis of biological tissues preserved close to their native state. *J Struct Biol* **2016,** *196* (3), 487-495.
4. Campbell, F.; Bos, F. L.; Sieber, S.; Arias-Alpizar, G.; Koch, B. E.; Huwyler, J.; Kros, A.; Bussmann, J., Directing Nanoparticle Biodistribution through Evasion and Exploitation of Stab2-Dependent Nanoparticle Uptake. *ACS Nano* **2018,** *12* (3), 2138-2150.
5. Arias-Alpizar, G.; Koch, B.; Hamelmann, N. M.; Neustrup, M. A.; Paulusse, J. M. J.; Jiskoot, W.; Kros, A.; Bussmann, J., Stabilin-1 is required for the endothelial clearance of small anionic nanoparticles. *Nanomedicine* **2021,** *34*, 102395.
6. Schledzewski, K.; Geraud, C.; Arnold, B.; Wang, S.; Grone, H. J.; Kempf, T.; Wollert, K. C.; Straub, B. K.; Schirmacher, P.; Demory, A.; Schonhaber, H.; Gratchev, A.; Dietz, L.; Thierse, H. J.; Kzhyshkowska, J.; Goerdt, S., Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors. *J Clin Invest* **2011,** *121* (2), 703-714.
7. Arias-Alpizar, G.; Kong, L.; Vlieg, R. C.; Rabe, A.; Papadopoulou, P.; Meijer, M. S.; Bonnet, S.; Vogel, S.; van Noort, J.; Kros, A.; Campbell, F., Light-triggered switching of liposome surface charge directs delivery of membrane impermeable payloads in vivo. *Nat Commun* **2020,** *11* (1), 3638.
8. Yun, S. H.; Kwok, S. J. J., Light in diagnosis, therapy and surgery. *Nature Biomedical Engineering* **2017,** *1* (1), 0008.
9. Anderson, E. J.; Rouphael, N. G.; Widge, A. T.; Jackson, L. A.; Roberts, P. C.; Makhene, M.; Chappell, J. D.; Denison, M. R.; Stevens, L. J.; Pruijssers, A. J., et al., Safety and Immunogenicity of SARS-CoV-2 mRNA-1273 Vaccine in Older Adults. *N Engl J Med* **2020,** *383* (25), 2427-2438.
10. Sahin, U.; Muik, A.; Derhovanessian, E.; Vogler, I.; Kranz, L. M.; Vormehr, M.; Baum, A.; Pascal, K.; Quandt, J.; Maurus, D., et al., COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. *Nature* **2020,** *586* (7830), 594-599.
11. Moitra, P.; Alafeef, M.; Dighe, K.; Frieman, M. B.; Pan, D., Selective Naked-Eye Detection of SARS-CoV-2 Mediated by N Gene Targeted Antisense Oligonucleotide Capped Plasmonic Nanoparticles. *ACS Nano* **2020,** *14* (6), 7617-7627.