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## Gaining control of lipid-based nanomedicine by understanding the nano-bio interface

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## Chapter 6

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### Summary and outlook

The overarching goal of the research presented in this thesis was to add fundamental knowledge, as well as exploit prior knowledge, to understand and manipulate lipid-based nanomedicine at the bio-nano interface. To this end, the work described herein has focused on two themes, specifically: 1. protein adsorption to lipid-based nanoparticles in biological media and 2. the improvement of RNA delivery selectivity and efficiency using ionizable lipid nanoparticles (LNPs). In order to achieve this, an ensemble of chemical, biophysical and biological methodologies was employed. This chapter will summarize the key discoveries, their implications for the field and provide an outlook on advancing the research topics.

## 6.1 The (un)importance of the protein corona

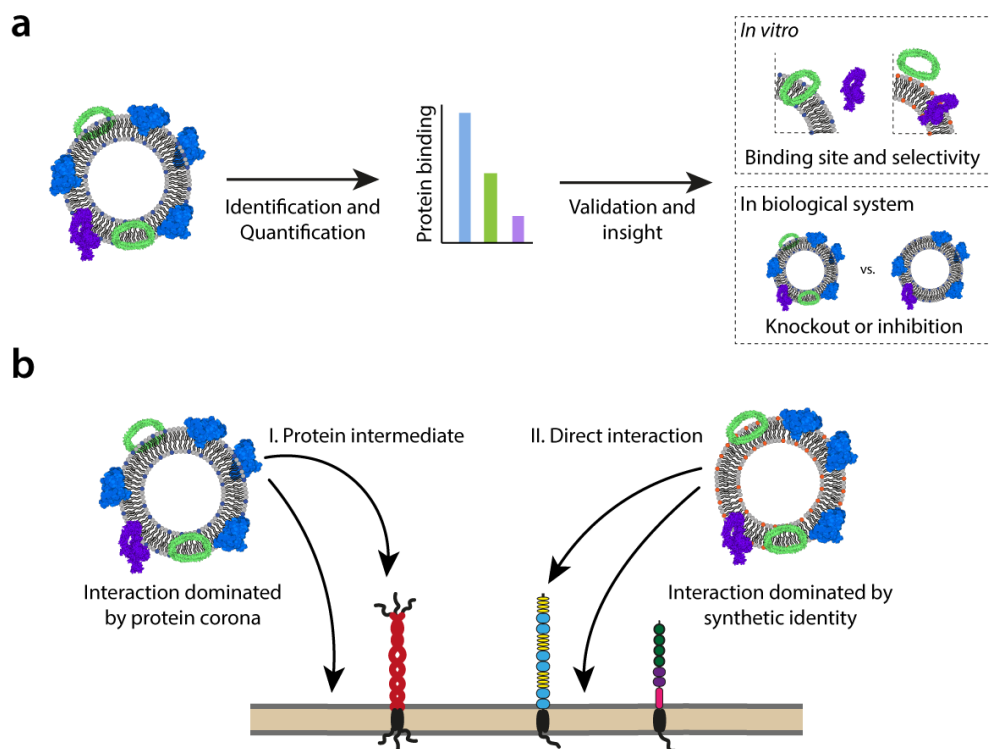
In **Chapter 2**, a new method for the determination of protein adsorption to liposomes in biological media was described. Here, a bifunctional photoaffinity based lipid was synthesized which allows for co-formulation in liposomal formulations and *in situ* crosslinking with adsorbed proteins to their surface through a photo-active diazirine moiety. Following this, the azide moiety allowed for click-chemistry conjugation with a fluorophore to visualize the subset of protein binding with gel-electrophoresis, or with a biotin group for the isolation of crosslinked lipid-protein complexes. The isolated proteins were digested and identified and quantified using label-free proteomics. Significantly, compared to physical isolation methods, this avoids the isolation and inclusion of highly abundant and/or heavy, unbound serum proteins.<sup>1-3</sup> This approach also allowed for the identification and validation of key individual liposome-protein interactions. In particular, validation of identified proteins hits is required to exclude false-positive identification, which was problematic using previous physical isolation methods.

**Chapter 4** expanded the work using the photoaffinity based method for protein corona determination towards LNPs encapsulating RNA, accurately identifying key individual protein-nanoparticle interactions that have a proven effect on the *in vivo* fate of LNPs. Generation of such a positive control served as an important milestone in laying the foundation of this methodology, and highlighted its potential for the use in mechanistic understanding of the protein corona. Furthermore, this method yielded more insights into key protein-nanoparticle interactions. In particular, using a competition assay it was shown that apolipoprotein E binds anionic LNPs site-selectively through its surface exposed heparin binding domain. The ability to uncover the molecular selectivity of a single protein to chemically-distinct nanoparticles is pivotal to elucidate protein selectivity, and provides another layer of depth to explain and predict the *in vivo* fate of nanoparticles.

As described in Chapters 2 and 4, it is possible that previous common protein corona determination methodologies can be intrinsically flawed, or lack the possibility for validation. Consequently, the research field regarding the protein corona may be dominated by false-positive data and hypotheses. In addition, the field is quickly transcending from a “protein” corona towards a “biomolecular” corona, involving the adsorption of other biomolecules (*e.g.*, polysaccharides, small molecules, lipids) to nanoparticles.<sup>4,5</sup> However, the importance of these interactions are still subject to heavy speculation. Therefore, more effort should be dedicated to developing unbiased identification methods and generating multiple lines of evidence for the validation of binding and the effect in a biological system (**Figure 1a**). To this end, further exploitation of the chemical biology toolbox used for small molecules to study nanomaterials at the bio-nano interface,<sup>6</sup> for example via chemoproteomic discovery and mapping of binding hotspots,<sup>7,8</sup> can serve as a useful methodology for further advancement of the field. Beyond the methodology, the obtained results from the chapters in this thesis give some important insights into protein corona formation on lipid-based nanoparticles, which can help shape future research in the field.

Firstly, the protein corona profiles reported in this thesis are typically dominated by apolipoproteins over more abundant serum proteins, which can be rationally explained by the evolved function of soluble apolipoproteins to bind lipidic surfaces of endogenous lipoproteins. As evolution has led to a carefully orchestrated network of protein-protein and protein-membrane interactions, the identification of exotic interactions that would inhibit endogenous functions should be carefully examined.<sup>9</sup>

Secondly, a dominant opinion within the field is that the protein corona is omnipresent for all nanoparticles and heavily impacts *in vivo* fate. In some cases, the protein corona is schematically drawn to fully decorate the nanoparticle.<sup>10</sup> However, these illustrations often lack experimental proof and can heavily bias the view of protein corona importance. In contrast, some of the protein corona profiles reported in this thesis show a near-to-complete absence of protein adsorption. Although the exact determinants of this have not been elucidated, it does provide evidence that in some cases it is not the protein corona, but the underlying synthetic identity of the nanoparticle that is interacting with cellular receptors or membranes.



**Figure 1. Important considerations for studying protein-nanoparticle interactions.** (a) Methods should focus on the correct identification of protein binding and allow for validation with multiple lines of evidence. In the case of similar protein binding, probing binding sites can facilitate deeper understanding of key protein-nanoparticle interactions (b) The presence of a protein corona might not be critical for the nanoparticle fate *in vivo*, and direct nanoparticle-receptor/membrane interactions should not be excluded.

For the future of the field, it is important that multiple scenarios of protein binding and implications for their *in vivo* fate are considered when validating results (**Figure 1b**). Firstly, in the scenario that the protein corona does indeed play a role in the cellular uptake, an intermediate function of these proteins with receptors, or the direct interaction with cellular membranes, has to be considered. Secondly, there might be scenarios where even dominantly binding proteins do not play a role in the cellular uptake of nanoparticles. In this case, the direct interaction of the nanoparticle surface with receptors, or a direct interaction with the cellular membrane has to be considered. Here, the above-mentioned topics regarding the validation of protein both *in vitro* and *in vivo* will play an important

role. Altogether, the understanding of the protein corona is complicated and heavy interdisciplinary, requiring a careful in-depth examination with the proper tools.

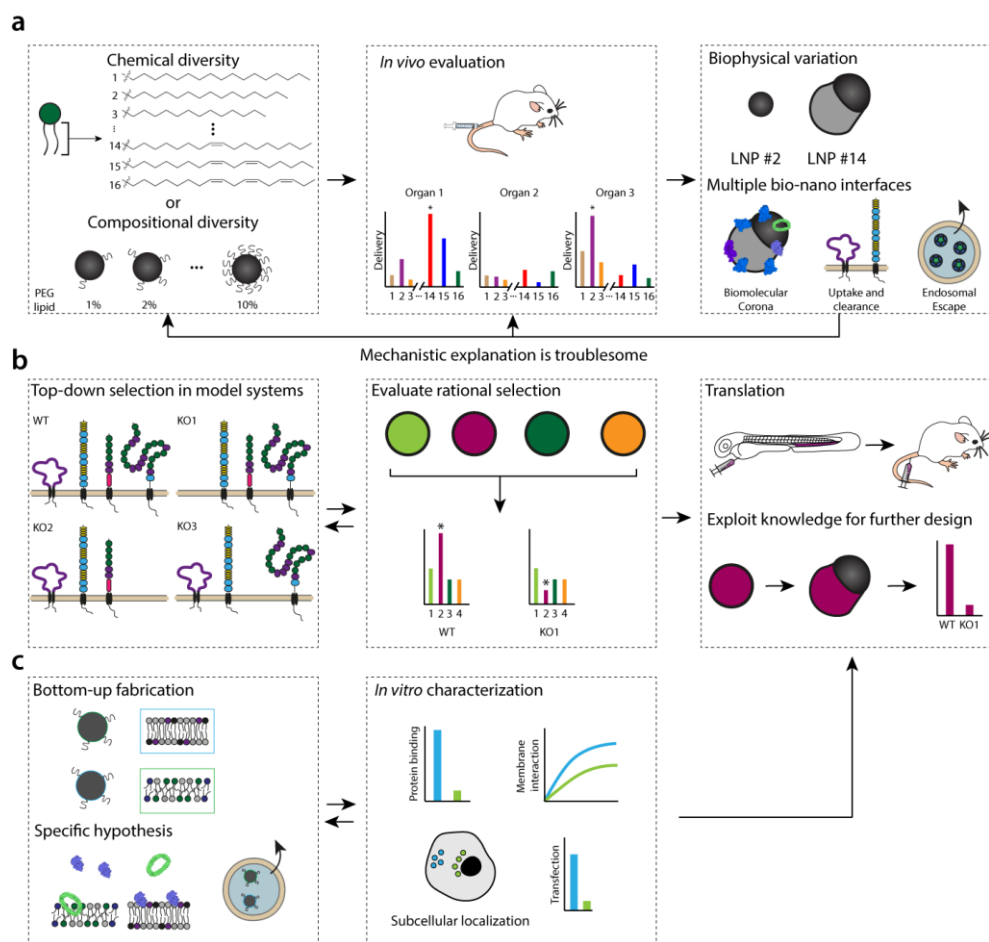
## 6.2 Lipid nanoparticle design and understanding

In **Chapter 3**, an anionic LNP formulation was designed and evaluated for the preferential delivery of mRNA to the reticuloendothelial system (RES). This strategy relied on prior knowledge regarding the receptor mediated clearance of anionic liposomes, as well as biophysical knowledge of lipid organization in multicomponent LNPs. The strength of this approach was the ability to redirect the delivery of mRNA from liver hepatocytes to the RES starting from a commercial LNP formulation, by the rational change of a single lipid in its composition. *In vivo* prescreening studies in embryonic zebrafish allowed for real time fluorescent imaging of biodistribution and transfection. Additional studies in genetically engineered embryonic zebrafish provided multiple lines of evidence regarding the receptor specific uptake of these LNPs into scavenger endothelial cells and macrophages. Finally, these data were supported by translational studies into mice, showing similar selectivity and effectivity of delivering mRNA to the RES.

**Chapter 5** described the fabrication of LNPs containing pre-programmed inverse hexagonal lipid phases encapsulating siRNA. Here, intrinsic properties of lipid mixtures were used to fabricate non-lamellar structures in LNPs for the first time. Using a variety of biophysical methods, including cryogenic electron microscopy and small-angle X-ray scattering, the fabrication of these structures was validated. In addition, the effect of lipid composition, RNA content and temperature on their formation, three-dimensional organization and stability was determined. The generation of LNPs with distinct internal lipid structures allowed for comparison of transfection efficiency. Furthermore, a mechanistic study on the interaction with endosomal model membranes suggests that inverted phases contribute to a more efficient one-step release mechanism by bypassing an initial lamellar to inverse hexagonal transition.

Together, these results demonstrated alternative approaches for LNP design, in which generating or exploiting bio-nano interactions play an important role. In general, the future of nanomedicine research would greatly benefit from more in-depth understanding of LNP fabrication and their interactions with different interfaces.<sup>11,12</sup> Common approaches for LNP development are typically empirical, relying on the generation of libraries, for example by chemically diversifying the components or through empirical formulation variation (**Figure 2a**). The direct *in vivo* evaluation is typically labour intensive and expensive, but can generate the selection of promising LNP formulations for efficient delivery to specific

cell types or organs. Nevertheless, mechanistic explanation in hindsight is troublesome due the size of the screen, large variation in biophysical properties between LNP formulations and obscurity to which bio-nano interfaces play a crucial role in their output. Therefore, alternative workflows should be employed for the mechanistic understanding and rational discovery of LNPs.



**Figure 2. Workflows for mechanistic understanding of LNPs at the bio-nano interface (a)** Common empirical approach based on the screening of LNPs makes retrospective mechanistic explanation troublesome. **(b)** Top-down selection of for example receptor targets coupled with rational selection of nanoparticles can gain useful information for further translation and new design. **(c)** Bottom-up design of LNPs and the determination of their effect on isolated bio-nano interfaces can help gain more understanding for translation and LNP design.

Firstly, the cellular uptake processes can be addressed systematically in a top-down approach (**Figure 2b**). Here, pre-existing knowledge on the uptake of endogenous nanoparticles through a variety of receptors (e.g., lipoprotein receptors, scavenger receptors) can be used to generate model systems that allow for determining their individual effects. A selection of nanoparticles with distinct chemical and biophysical properties can aid in the validation of the effect and selectivity of these processes. The selection of nanoparticles and receptor targets works bidirectionally, in which information from one screen can help select targets in the other. Finally, once dominant effects are obtained, this knowledge can be used for further translation into other models, or for the design of other nanomedicine that will work through the same mechanism.

Secondly, the influence of LNP components and biophysical characteristics on specific bio-nano interfaces can be studied (**Figure 2c**). Herein, bottom-up fabrication of LNPs with defined characteristics has to be coupled with extensive characterization. The selection of a specific hypothesis (e.g., protein binding, endosomal escape) allows for the use of isolated *in vitro* experiments and leads to stronger conclusions about the effect on individual bio-nano interfaces. Finally, this knowledge can be used for further translation of specific formulations, or be exploited for further design. Altogether, the field of LNP-based RNA therapeutics holds great promise for the treatment of a widespread of diseases. One of the ways of unlocking this potential is by relying more on systematic approaches of their mechanistic behaviour, besides solely empirical screens.

### 6.3 References

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