Summary and conclusions

Since its discovery, small interfering RNA (siRNA) therapeutics sparked high hopes for the treatment of a wide variety of diseases including cancer, viral infections and genetic diseases. The advantage of siRNA as therapeutics is represented by the ability to target 'undruggable' pathologies that are characterized by the overexpression of particular genes. Despite the unquestionable advantages, the clinical translation of such molecules has been slowed down by the instability and unfavorable pharmacokinetics and biodistribution. To overcome these issues, delivery vectors have been developed to protect siRNA and deliver it through the myriad of biological barriers. In this regard, both viral and non-viral vectors were investigated to ensure siRNA packing and intracellular uptake. Non-viral vectors have emerged as an interesting delivery system because of their ease of production, increased safety and tailorable physicochemical features, compared to viral alternatives. Despite their numerous advantages, non-viral vectors mediated intracellular delivery is still limited by the major bottleneck related to their endosomal entrapment. Even for state-of-the-art nanocarriers, only a minority of the internalized dose can reach the cytosol while the major bulk is degraded following the endolysosomal degradation pathway. Therefore, several strategies have been explored to improve endosomal and lysosomal escape.

In **Chapter 1**, we provided a general introduction to the use of siRNA-based therapeutics and an overview of the different types of non-viral vectors developed over the years to enhance the intracellular delivery of siRNA. After describing the relevant characteristics of nanoparticles (NPs) we discussed their reported applications. Then, we discussed the endosomal escape mechanisms characterized so far. Subsequently, we illustrated the interactions between NPs and endosomal membranes and we briefly discussed the methodologies applied to visualize and quantify such interactions.

Systemic administration of NPs is limited by their clearance in certain organs such as the liver. However, local administration can offer several benefits to reach other tissues as well (e.g. need for a lower dose, facilitated targeted delivery and thus lower risk of side effects, direct contact with target cells, etc.). In this context, the lungs represent a particular target organ for local drug delivery. In the context of pulmonary drug delivery, the use of pulmonary surfactant (PS) is gaining interest. In **Chapter 2**, we conducted a literature study to investigate the use of PS and specific surfactant proteins (SPs) as biomaterials for (pulmonary) drug delivery. Recently, several research groups reported on the actual benefits of using biomaterials as *in vivo* transport vehicles for therapeutics, to combine increased biocompatibility with potential endogenous targeting. In this respect, PS is a particular biomaterial that has been proven to promote both the diffusion of drugs into the lungs both the intracellular delivery of NPs and their cargo. In addition, in this chapter we described the unique profile of SPs and their impact on the different steps of the drug delivery process, including modulation of pulmonary distribution, cellular targeting and intracellular delivery. More specifically, we illustrated the effect of the specialized lung surfactant protein B (SP-B) on the improved siRNA delivery of proteolipid-coated hydrogel nanoparticles (i.e. nanogels), a hybrid nanocomposite developed in our siRNA inhalation therapy research group. Encapsulating the siRNA-loaded nanogels (siNGs) with a proteolipid composition including SP-B has been shown to improve not only the colloidal stability of the NPs, but also the cytosolic siRNA release from such nanocomposites *in vitro* and *in vivo*.

Since the beneficial effect of PS and SP-B on the intracellular release of siRNA is a fairly recent finding, in **Chapter 3** we tried to investigate those factors affecting (positively and negatively) SP-B mediated intracellular delivery. Initially, we demonstrated an identical beneficial effect of PS on siRNA delivery in extra-pulmonary cell types, suggesting that the beneficial role of SP-B is not limited to lung cells and that its application could potentially be extrapolated to other target organs and other administration routes. In addition, we examined in more detail the influence of the various components of the hybrid nanoformulation on the functionality of SP-B, revealing a crucial role of the surrounding lipid membranes. Since the role of SP-B in the alveolar space is highly dependent on its combination with the PS-specific lipids, these data confirmed a possible analogy between the SP-B mode-of-action at the alveolar interface and the one at the intracellular level.

The SP-B mechanism of action responsible for the reduction of surface tension at the alveolar interface has been extensively described. SP-B's positive effect on the intracellular siRNA delivery of proteolipid- coated nanogels has been previously reported by our group, but the mechanistic insights have remained unclear to date. In **Chapter 4** we aimed to gain a deeper insight into the (intra-) cellular interactions of SP-B, responsible for the observed improved siRNA delivery. Using advanced microscopic techniques, including spinning disk confocal microscopy and focused ion- beam scanning electron microscopy (FIB-SEM), we were able to (1) visualize the cytosolic release of siRNA and (2) demonstrate the impact of SP-B on the intracellular distribution of SP-B containing liposomes. These experiments confirmed previous data indicating that SP-B has no (beneficial) effect on the intracellular uptake of the siRNA- loaded nanocomposites, but rather that the protein is active at the endolysosomal level. Detailed membrane fusion assays showed a strong correlation between the pH-independent SP-B mediated fusion with anionic endosomal membranes and cytosolic siRNA delivery, a mode-of-action resembling those of certain lipid-enveloped viruses and so-called cell - penetrating peptides (CPPs). Building on these insights, we were able to further optimize the SP-B proteolipid composition, which led to a substantial improvement in the intracellular siRNA delivery.

The use of SP-B as an adjuvant for siRNA delivery represents a unique approach in repurposing an endogenous material (i.e., PS) for drug delivery. However, the wide application of SP-B as an intrinsic component of nanomedicines presents additional challenges. The exact 3D structure of SP-B has not been defined experimentally and neither the SP-B obtained by chemical synthesis or recombinant synthesis succeeds in mimicking the membrane-specific interactions of the native protein. Therefore, the only current method to obtain SP-B is via extraction and purification from PS of animal origin, which however is a limiting factor in the clinical use of SP-B based nanocarriers. For this reason, in **Chapter 5** we tested different SP-B derived synthetic peptides. More specifically, this chapter focused on the Mini-B (MB) and Super Mini B (SMB) peptide described in the literature, which differ only for the presence of the hydrophobic N-terminal heptapeptide segment. Remarkably, only the SMB peptide (in which this specific sequence is present) was able to mimic the beneficial effect of SP-B on siRNA delivery, both in proteolipid- coated nanogels and in simpler lipid nanoparticle (LNP) complexes. Of note, previous experiments with a synthetic peptide (i.e. KL4) that mimics the C-terminus of the SP-B protein did not result in a positive effect on gene suppression, which further confirms the importance of the N-terminal domain in endosomal escape processes and cytosolic siRNA delivery. The discovery of the endosomal escape activity of the synthetic SMB peptide offers interesting opportunities for the future development of PS-inspired and siRNA-loaded nanocarriers.

Finally, in **Chapter 6** we provide the broader international context of this work and critically discuss the prospects of this research domain. This chapter first discusses the most important milestones of RNAi based therapeutics, from the discovery of RNAi mechanism to the recent clinical advance of siRNA-based drugs. Although the lung is an extremely interesting target for RNAi inhalation therapy, the lack of therapies in clinical trials further suggests the need for the development of more advanced delivery vectors. In this context, we propose that gaining a deeper understanding of the interactions occurring at the nano- bio interface, specifically in the lungs, could greatly help the development of RNAi inhalation therapy in the clinic.