6.1 Summary

The malfunctioning of proteins often has an impact on cellular activity in that disturbances may occur in cellular functioning and metabolism. As a consequence, impairment of physiological functions may take place, leading to diseases. Defects in one or more proteins, resulting, for example, from mutations in their genes, can trigger proliferative signaling, cause resistance to cell death, or induce invasiveness, all of which are processes that may trigger the development of cancer. Basic cancer therapy often relies on treatment with cell-killing or cell-growth inhibiting chemical substances, also known as chemotherapy. However, chemotherapeutic agents are commonly not specifically targeted to the rapidly dividing cancer cells, implying that normal cells are also affected, thus giving rise to significant side effects. Another cancer treatment regime, still in development, concerns gene therapy, which relies on the reintroduction of a functional gene or the silencing of an ill functioning gene that gives rise to the cancerous state. Yet, irrespective of the chosen therapeutic strategy, reintroducing a functional gene, silencing a dysfunctional protein or inducing cell death by applying chemotherapeutic agents, the success rate of the treatment also very much depends on the ability of the ‘drug’ to efficiently reach intracellular targets. Thus, both chemotherapy and gene therapy would greatly benefit from a more accurate (intra) cellular delivery of the ‘drug’, so as to eliminate side effects of chemotherapy and increase the efficiency of gene therapy.

To achieve such goals, sophisticated delivery devices or ‘nanoparticles’ are currently developed and applied in order to effectively and specifically guide the therapeutic agents to their targets. In this manner, potential therapeutic genes are protected against degradation in the blood while encapsulated chemotherapeutics can in principle be precluded from entering ‘healthy’ cells. A great variety of nanoparticles are currently evaluated for such applications, including so-called Solid Lipid Nanoparticles (SLNs). These particles are biocompatible, physically stable, easily allow for large-scale production, and have been shown of being able to deliver drugs and/or genes into cells. In this thesis we have investigated the potential use of SLNs as a tool to improve intracellular delivery of genes and
drugs in the treatment of cancer. Specifically, detailed studies are presented on the interaction of solid lipid nanoparticles with and their processing by normal prostate and prostate cancer cells in vitro.

In Chapter 1 we address recent advances in the use of Solid Lipid Nanoparticles as platform for delivery of nucleic acids as therapeutic agents. In particular, we focus on underlying molecular mechanisms by which SLNs and nucleic acids assemble into complexes, and how the nucleic acid cargo may be released intracellularly. Thus, we developed a coherent model of the interaction between SLNs and nucleic acids and pointed out the key differences between the intracellular release mechanisms of cationic lipid-based liposomes and SLNs as gene delivery devices. Thus, a concise and critical overview is provided of the knowledge on the use of SLNs for gene delivery, which appears fairly limited so far, when compared to mechanistic knowledge on the use of other nanocarriers, including those based on (cationic) polymers and cationic lipids. Accordingly, further successful development of SLNs as gene delivery vehicles will greatly depend on the improvement of knowledge on structure-function relationships of SLNplexes and, consequently, the underlying mechanisms of intracellular processing.

The first experimental challenge of the work presented in this thesis was the development of a convenient and versatile procedure for the production of SLNs on a laboratory scale. In Chapter 2 we demonstrate that an approach relying on the use of a mini-extruder can be advantageously employed to produce solid lipid nanoparticles on a laboratory scale. The described microemulsion extrusion technique is fast, inexpensive, reproducible, free of organic solvents, and suitable for small volumes. In addition, the procedure provides a most efficient means of screening for new formulations of lipid-based carriers, including solid lipid nanoparticles and nanostructured lipid carriers. Additionally, we show that the produced lipid nanoparticles are stable and versatile, being applicable in both drug and gene (i.e., nucleic acid) delivery. In fact, the co-delivery of the chemotherapeutic drug mitoxantrone and a plasmid, containing the PTEN construct, successfully reduced the viability of PC3 prostate cancer cells and bypassed the resistance of MCF-7 breast cancer cells to mitoxantrone.

After having established the production of SLNs, using the microemulsion extrusion technique, efforts were undertaken to improve the SLN-mediated delivery of genes into PC3 prostate cancer cells. Guided by the notion that distinct helper lipids like DOPE may facilitate the formation of non-bilayer lipid phases, leading to an enhanced destabilization of the endosomal membrane and, as a consequence, improved cytosolic delivery of the plasmid and subsequent transfection efficiency, the effect of inclusion of DOPE in the SLN formulation was investigated to evaluate this hypothesis. Unexpectedly, as opposed to DOPE’s ability to strongly promote transfection, mediated by cationic liposomes, we observed that in-
clusion of DOPE into the solid lipid nanoparticles has a detrimental effect on the gene delivery efficiency of SLNs (Chapter 3). We show that DOPE in SLN formulations composed of stearic acid/DOTAP/Pluronic-F68 compromises DNA-binding and leads to inefficient protection of DNA against DNase degradation. On the other hand, SLNs devoid of DOPE are shown to efficiently transfect PC3 cancer cells, and maintain their high transfection efficiency after lyophilization and long-term storage, an important asset for biomedical applications. These findings show the potency of developing SLNs for gene delivery purposes. They also indicate differences in the mechanism of delivery, when compared to the mechanism of delivery mediated by cationic lipid systems, reflecting interesting differences between both carrier systems that warrant further investigations.

Following the above findings, we subsequently investigated potential cell type dependent differences in transfection efficiency by careful examination of the interaction of a given SLN formulation with both normal prostate cells and their cancerous counterpart. In Chapter 4 we demonstrate that SLNs are efficiently internalized by human prostate cancer PC3 cells, as well as their non-malignant counterparts, i.e., human prostate epithelial PNT2-C2 cells. However, PC3 cells become efficiently transfected, whereas PNT2-C2 cells do not. This is in sharp contrast with transfection mediated by Lipofectamine, that reaches similar efficiencies in both cell types. We show that although SLN lipoplexes induce autophagosome formation in both PC3 and PNT2-C2 cells, this phenomenon is connected with autophagic degradation in PNT2-C2 cells, but not in PC3 cells, which showed an incomplete autophagic process. Therefore, we suggest that a low autophagic flux in the PC3 tumor cells compared to that in non-malignant PNT2-C2 cells broadens the time window for effective endosomal escape of cargo, resulting in its selective and effective release into the tumor cells, but not their healthy counterparts. We submit that this finding can be exploited for the ‘targeting’ of gene delivery vectors into cancer cells. Knowledge about the underlying mechanism of transfection can be exploited to improve transfection efficiency; here we show that this can be achieved by taking advantage of how the cells process nanoparticles. Therefore a thorough understanding of endocytosis, cell signaling and intracellular processing in cancer cells, especially upon interaction with (gene) vectors, will aid in the rational design of gene/drug delivery systems.

In Chapter 5 we studied the effect of ferruginol, a natural compound found in Chilean trees (Podocarpaceae), on prostate cancer cells. We found that this diterpene reduced cell viability and was capable of inducing apoptosis in PC3 cells. Interestingly, ferruginol triggered both intrinsic and extrinsic apoptosis pathways in prostate cancer cells. The induction of intrinsic pathways was characterized by the high ratio of Bax:Bcl2, high nuclear AIF levels and caspase 9 activity. Induction of an extrinsic apoptotic path-
way was confirmed by the high expression of TNFR1 and a high activity of caspase 8. Accordingly, ferruginol-treated cells increased caspase 3 activity, a converging point for both pathways. Besides induction of apoptosis, ferruginol was able to inhibit survival-signaling pathways (e.g. PI3k/AKT and MEK/ERK pathways) and proliferation. Finally, we demonstrated that ferruginol reduced cell survival by inhibiting Hsp27, which in turn destabilizes and inhibits STAT3. The observed effectivity of the free drug on the prostate cancer cells are encouraging in further developing the system in terms of the application of SLNs to achieve targeted delivery and an effective anti-tumor therapy.

6.2 Perspectives

With the research presented in this thesis, we have obtained insight into the options of applying solid lipid nanoparticle as delivery vehicles, including knowledge of the mechanism of delivery, their intracellular processing and the cellular response. We demonstrated that lipid-based nanoparticles, including SLNs and nanostructured lipid carriers can be conveniently produced by a mini extruder according to a method that we refer to as ‘the microemulsion extrusion technique’. It would be of future interest to examine whether this method could be readily translated into scale up methods, such as a high pressure homogenization technique. In particular, the currently developed procedure allows the preparation of SLNs on a small volume scale (ml’s), which enables a rapid screening for potentially successful formulations, prior to a relatively large scale production, which is commonly an inherent part of existing procedures, as they preclude the use of small volumes.

Further, it would be important to better define the physicochemical characteristics of the internal molecular organization (the ‘core’) of the lipid nanoparticles. Recently, numerous SLN formulations have been evaluated as gene delivery systems. Several of those formulations contain a cationic phospholipid (e.g. DOTAP) or helper lipid (e.g. DOPE). However, inclusion of liquid crystalline lipids (e.g. the transition temperature of DOTAP is -11.9 °C and that of DOPE is -20 °C) into SLNs that primarily consist of a solid core, seem to declassify these particles as solid, and should rather be considered as nanostructured lipid carriers (NLCs); particularly since it should be noted that this simple modification may have an important impact on the lipid nanoparticle structure, and hence its functioning. NLCs represent the new generation of lipid-based nanoparticles, which show some advantages over SLNs, such as the capacity of modulating drug release, an increase in drug loading capacity and a reduced cargo leakage. Unfortunately, up to now this discussion has been neglected in the SLN literature. In fact, this example illustrates that often only poor attention is paid to details of the
structure of the lipid nanoparticles, which is nevertheless of relevance for a better understanding of their properties as a delivery system. Furthermore, it is also most important to shed further light on the underlying mechanisms of nucleic acid release from the nanoparticles, since such knowledge will be essential in further improving the delivery properties and hence, the transfection efficiency.

The cellular uptake of SLNplexes (the complex that arises upon mixing SLN particles and the gene of interest) via endocytosis depends on their binding to the cell membrane. Typically, positively charged nanoparticles have been thought to efficiently bind to the net negatively charged cell membrane, which consequently results in the internalization of the nanomaterial. Yet, SLNplexes are highly negatively charged nanomaterials that are effectively internalized by prostate cancer cells. Thus, unlike previous assumptions that only positively charged particles efficiently interact with the plasma membrane, our data suggest that the interaction between cells and nanomaterial may not be solely based on electrostatic interactions. Future investigations should provide more insight into alternative mechanisms of SLN-cell surface interactions and the consequences these characteristic may have on in vivo applications. After internalization via the endocytic pathway, the cargo becomes entrapped in endosomes, which is eventually processed towards lysosomes, where lysosomal enzymes may proceed with its active degradation. Escape from the endosome is thus mandatory for a successful intracellular delivery of nucleic acids; however, the ultimate molecular mechanisms by which SLN-mediated endosomal escape of its cargo (such as nucleic acids) occurs, remains puzzling. Thus, concerning the mechanism of transfection, studies of intracellular processing and fate of SLNplexes should aim at providing improved insight into the mechanism of nucleic acid release. Because in sharp contrast to cationic liposome-mediated transfection, the transfection properties of SLNs do apparently not depend on lipid mixing or nonbilayer structures that potentially may destabilize endosomal membranes, thereby facilitating release. Important molecular factors that govern the release of cargo from SLNs therefore remain to be discovered. In this context, SLNplexes are shielded by DNA, which may hamper lipid exchange between SLN and the endosomal membrane. The inability to adopt hexagonal conformations and the detrimental effect of the inclusion of a hexagonal phase promoting helper lipid (e.g. DOPE) show a key idiosyncratic difference between SLN and cationic lipid-based liposomes. However, these findings trigger additional questions that need to be addressed, such as: Do SLNs need to be disassembled to allow for payload release? Which forces drive this event? Clearly, further insight into the structure-function relationship of these particles is crucial.

Autophagy has emerged as a mechanism related to nanomaterial toxicity. Recently, autophagy was suggested as a new cellular barrier against
efficient gene delivery, because cationic nonviral gene delivery vectors promptly induce the formation of tubulovesicular autophagosomes. In showing that cancer cells are more efficiently transfected by SLNs, when compared with normal cells, we were able to correlate this difference with differences in the process of autophagy in normal prostate cells versus cancer prostate cells. The incomplete autophagic process in prostate cancer cells thus seems to offer a ‘natural approach’ for improved delivery of nucleic acids into prostate cancer cells. It would be of particular interest to determine whether these findings apply to cancer cells in general, and to identify the underlying molecular causes of this effect. Finally, it would also be of great value for therapeutic purposes to investigate if this intriguing difference between normal and cancer cells can be explored for \textit{in vivo} delivery of therapeutics, including nucleic acids and drugs.