Summary and Perspectives

The research described in this thesis investigated new drug delivery strategies designing surface modified nanocarriers based on mesoporous silica, able to control the release kinetics of guest molecules and to deliver hydrophobic and hydrophilic compounds *in vivo*. The delivery of drugs in a local defined environment is becoming nowadays a crucial point in the development of new therapies because of several side effects associated to the systemic treatments, which can impair the patient quality of life.

The mesoporous silica nanoparticles (MSNs) were designed to meet several prerequisites for an effective drug delivery system (DDS). First the DDS has to protect the payload from enzymatic degradation which occurs in the blood circulation in order to be able to deliver the maximum amount of active compound in the targeted site. Second the DDS has to avoid any premature release to minimize the side effects ideally and it has to deliver the cargo on demand. In addition the silica nanoparticles have a large surface area due to their inner mesoporosity which allows the incorporation of large quantities of a drug, and have an inner and external surface which can be functionalized independently and easily to create nanovalves.

In this thesis mesoporous silica nanoparticles were designed towards the deliver of chemical compounds *in vivo* in two different manners. Initially MSNs were functionalized on the external surface exploiting the nanovalve strategy in order to control the release of guest compounds. The nanovalve system consists of a monolayer of mechanically interlocked molecules (i.e. the rotaxane) composed of: a linear stalk anchoring the rotaxane to the surface, a gating ring which encircles it and locks the cargo inside the mesopores, and a stopper located at the end of the linker connected to it with a cleavable bond.

In the second part of this thesis, silica nanoparticles were applied as *in vivo* delivery system for hydrophobic drugs. These particles were not modified with nanovalves, but the surface was homogeneously covered with polyethylene glycol chains to lower the immune response.

In **Chapter 1** an overview of mesoporous silica nanocontainers as potential drug delivery systems and their interactions with cellular environment is given. In **Chapter 2** the design and the synthesis of peptide modified mesoporous silica nanocontainers (PMSNs) is shown. PMSNs are a snap-top system where the terminal peptide acts as a

nanovalve activated via reduction of disulfide bond by the action of glutathione (GSH) and the cyclodextrin has the role to prevent undesired release of the guest molecules. The terminal peptide is 13-mer oligopeptide of the HIV-1 TAT peptide virus membrane protein which is responsible for translocation of exogenous molecules across the plasma membrane. The high affinity of this peptide to the membrane proteins allows the HIV-1 virus to easily penetrate in the immune cells. Therefore, the TAT peptide was chosen to increase the affinity between the nanoparticles and the cellular membrane of cancer cells. Flow cytometry analysis confirmed the tendency of PMSNs to be efficiently incorporated into cells compared to unmodified MSNs. This observation was proved with confocal scanning laser microscopy, showing that the PMSNs were located inside the cytoplasm of HeLa cells. In this chapter a detailed description of fluorescein release from PMSNs is given as a function of the rotaxane structure. The influence of the cyclodextrin on the release characteristics was studied and the crucial role in preventing aggregation in aqueous media was shown.

PMSNs show to be interesting nano-delivery systems, as the cell uptake is significantly enhanced by the presence of TAT peptide. In this system the endocytosis is actively driven by the presence of the peptides at the surface which enhances the interaction with the cellular membrane. This feature renders the nanosystem intriguing in comparison with several nanoparticles synthesized so far in which the cellular uptake is governed by passive endocytosis.

However the high affinity of TAT peptide for cellular membrane abolishes any selectivity towards specific type of cells by the carriers. Hence in **Chapter 3** the synthesis and characterization of MSNs targeted towards cancer cells are described. MSNs with a nanovalve system as described in **Chapter 2** were modified with folic acid. The rotaxane system was composed of an aliphatic stalk anchored to the surface of the nanoparticles, on which a cyclodextrin was tethered to avoid any uncontrolled leakage of the payload. In order to keep the cyclodextrin in place, a folic acid moiety was coupled to the stalk. This system is activated in cells by esterase hydrolysis of the ester bond in the rotaxane resulting in release of drugs. Enhanced uptake using folic acid modified MSNs was shown as folate receptors are overexpressed in more than 40% of human tumors.

The release kinetics of a model drug was studied in depth. The nanovalve responds to the action of the esterases resulting in a release of the content. Moreover, no leakage of the model drug was observed when the MSNs were dispersed in medium in absence of enzymes.

The nanoparticles were visualized after cell uptake using confocal laser scanning microscopy in order to localize the MSNs within the cell. The presence of the nanoparticles was confirmed both in the cytoplasm as well as in the nucleus of the cancer cells. This is of interest as these MSNs seem to exploit the presence of nuclear folate receptors resulting in internalization in this cell compartment. Thus these folate modified MSNs showed a controlled release of guest molecules and targeted delivery of compounds only in cancer cells.

To investigate the release of an active drug we used folic acid modified mesoporous silica nanoparticles were loaded with the anti-cancer drug camptothecin and the release was studied by observing the biological effects. A TUNEL staining assay and a Western Blot were performed in order visualize apoptosis caused by the drug release in the cellular environment. Upon activation in the cell, camptothecin is released in the cytoplasm and we observed that 90% of treated cells showed activation of the apoptotic pathway. Thus this delivery system is able to target cancer cells and releases effective amounts of the drug in the cellular environment.

The delivery in *Xenopus laevis* of hydrophobic compounds from mesoporous silica nanoparticles is described in **Chapter 4**. When exogenous particles are injected in living animals the main side effect is the development of an immune response which ultimately could lead to lethal consequences. Therefore mesoporous silica nanoparticles modified with polyethylene glycol groups were synthesized to minimize the immune response and to prevent clearance from the living environment. The expected protection is due to flexibility of the PEG chains and the hydrophilic shield resulting in a stable colloidal suspension. Moreover, pegylation of the surface prevents the process of opsonisation, a strong immune response with a cascade of events involving antibodies and leading to a fast phagocytosis of the nanosystem introduced.

While unmodified MSNs showed massive aggregation, PEG-modified nanoparticles remained well dispersed in buffered solutions. The release of drugs from these particles was investigated *in vitro* and with *in vivo* studies in order to confirm the delivery of the drug in significant amounts inducing a biological effect. In this study, retinoic acid was chosen as model compound. Retinoic acid (RA) plays a crucial role during morphogenesis and organogenesis of vertebrate species. Hence, excess of RA resulted in deformation of bones and organs due to an uncontrolled and chaotic expression of genes at sensitive stages where the development involves the activation of specific receptors which interact with RA. Malformations caused by RA release from PEGylated MSNs in the early stages of

development in *X. laevis* were shown with *in situ* hybridization techniques and with morphological analysis of the embryos. We studied the expression patterns of four genes: *Dll-3*, *Dll-4*, *Krox-20* and *Otx-2*. The retinoic acid release was confirmed as different patterns in the expression were observed as compared to the control siblings. This study showed that MSNs are effective delivery systems for hydrophobic compounds *in vivo* as the expected biological effects due to the release of the guest compounds was observed.

These results were confirmed by investigations performed in *Danio rerio*. In **Chapter 5** the biocompatibility of silica nanoparticles in zebrafish embryos was shown. Again RA was used as a model compound in order to compare these results with the study described in **Chapter 4**. Morphological and genetic studies in zebrafish embryos were performed after the injection of RA-loaded MSNs. The biocompatibility of the PEG-modified silica nanoparticles was shown as embryos injected with these MSNs developed with a normal phenotype. However, abnormalities in the development were observed in embryos injected with retinoic acid loaded nanocarriers. This observation was confirmed by genetic investigations performed with *in situ* hybridization of the *msxB* gene.

In **Chapter 6** the application of silica nanoparticles as emulsion stabilizers (i.e. Pickering emulsions) was investigated. The prepared colloidosomes had a crosslinked core in order to have an intrinsic resistance allowing further manipulations. Three types of colloidosomes were synthesized in which different nanoparticles were used: mesoporous silica nanoparticles, co-condensed mesoporous silica nanoparticles and non-porous silica nanoparticles. The release kinetics of fluorescent dyes from the colloidosomes as a function of the external silica layer were studied and a correlation between the shell composition and the drug release profile was observed. Colloidosomes stabilized with mesoporous silica nanoparticles showed to have an initial burst release of the payload while colloidosomes made with a non-porous shell had a delayed release of the cargo. Moreover the synthesis of a double drug release system was investigated in which the external shell was composed of modified nanoparticles while the core was loaded with a guest molecule. It was possible to observe a delayed release of the core cargo which started after the total depletion of the external layer functional groups.

A potential application of these drug delivery systems is as a drug release implant in the caudal fin of adult zebrafish. Microcapsules loaded with RA were implanted and a delay in the tail regeneration was observed. Morphological and genetic analysis on the tail portions and revealed a significant delay when a RA-microcapsule was implanted.

In conclusion the studies described in this thesis have contributed to the validation of mesoporous silica nanoparticles as drug delivery system in living environment. Several silica-based drug delivery systems were synthesized with controllable release kinetics and the MSNs showed to be biocompatible. While TAT-peptide functionalization enhances the cellular uptake in cancer cells, folic acid modification of MSNs led to target specific nanocarriers. Folic acid modified silica nanoparticles represented an evolution of the system shown in **Chapter 2**. The cellular uptake of these nanoparticles is enhanced using a folic acid which is the natural ligand of folate membrane receptors; moreover the target selectivity is achieved as folate receptors are overexpressed only on the surface of tumor cells. We envision a broader study of this system in living animal models in order to study potential applications as drug delivery system.

In addition we have validated these drug delivery system *in vivo* using two different models: *Xenopus laevis* and *Danio rerio*. The biocompatibility of peptide or folic acid surface modified silica nanoparticles was established in a cellular environment while polyethylene glycol functionalized nanoparticles were chosen as good candidate for *in vivo* studies due to high biocompatibility. The mesoporous silica nanoparticles synthesized have the ability to deliver significant amount of compound in order to show biological effects *in vitro* and in *in vivo* systems. Thus, MSNs can be used as delivery systems to improve pharmacological studies on the effect of hydrophobic drugs in aquatic animals, which so far have been always very limited due to the poor solubility of the drugs.

Finally, we have shown the potential application of local implantable beads for drug delivery using *in vivo* studies. The layer of MSNs on the surface renders these beads highly biocompatible reducing significantly immune response, while it allows the controlled release of the cargo depending on the particle modifications. In this study hydrophobic drugs were investigated, however we envision that a wider range of compounds can be tested with this system also in other animal models.

Our data will now enable in depth follow-up studies that will give new insights in the development of new drug delivery system for potential application in new therapies.