Summary and general conclusions

The main aim of this dissertation was to improve the selective delivery of chemotherapeutic agents to tumors by means of degradable targeted nanoparticle delivery systems and hence overcome some of the main limitations of cytostatic drugs. Furthermore, the concept of immunogenic cell death (ICD) was explored, which harnesses the immunogenic properties of chemotherapeutic drugs. Immunochemotherapy, which involves the combination of immunotherapy and chemotherapy, was investigated with the purpose to further enhance clinical efficacy and broaden therapy-responsive patient population.

Chapter 1.1 provides a concise overview on the discovery and mechanism of action of chemotherapeutics agents, along with the current treatment modalities for cancer applied in the clinic. In addition, the chemotherapeutic drugs that have been used in this PhD dissertation have been reviewed more in detail. A second part of this chapter highlights the potential of nanoparticle delivery systems to overcome the shortcomings of conventional chemotherapeutic treatment. In particular, nanoparticle systems may alter pharmacokinetic profile and improve therapeutic index of conventional chemotherapeutic drugs. Subsequently, the most striking features of cancer nanomedicine, which include passive and active targeting, are elucidated. Finally, the implementation of stimuli-responsive properties, which allow on-demand drug delivery and polymer degradability, are discussed. Among these stimuli-responsive stimuli, (transiently) thermo-responsive and pH-responsive polymers are outlined and underscore the versatility and specificity of the triggered nanoparticle platform.

In **Chapter 1.2**, the complex interplay between cancer progression and the immune system is discussed, which has led to the discovery and development of various immunotherapies. Next, the potential benefits of combining immunotherapy with chemotherapy have been thoroughly discussed. In particular, the concept of immunogenic cell death (ICD), which covers the underlying immune-modulating properties of certain chemotherapeutic drugs have been unraveled. A brief overview of the most important damage-associated molecular patterns (DAMPs), which have been discovered to date, and their characteristics are provided. Moreover, the underlying signal cascade transduction pathway for calreticulin (CRT) exposure,

which involves ER stress is also discussed more in detail. Finally, an overview and classification of the currently known ICD inducers are depicted along with the currently used *in vivo* screening procedures.

In **Chapter 2**, we proposed a straightforward route for the hydrophobic modification and purification of a HPMA derivative that yields degradable temperature-responsive polymers with benign degradation byproducts. By grafting an ethyl moiety to the pending OH group of HMPA via a hydrolytically sensitive carbonate ester, we showed that this monomer could be polymerized by free radical polymerization using a PEG-macroinitiator strategy. These block copolymers formed micellar nanoparticles in aqueous medium with a diameter of around 40 nm and exhibit a cloud point (cp) of 17°C, which is within an attractive range for drug delivery purposes. Indeed, simple heating above their phase transition temperature leads to self-assembly and the formation of hydrophobic domains that can be exploited to solubilize hydrophobic drug molecules, as illustrated in this work for paclitaxel (PTX). The formed micelle formulation showed substantial colloidal stability with a low critical micelle concentration (CMC). Under accelerated basic conditions, the nanoparticles decomposed into soluble unimers, which was evidenced by dynamic light scattering (DLS). In addition, it was demonstrated by flow cytometry (FACS) and confocal microscopy that the designed nanoparticle was able to deliver encapsulated hydrophobic compounds to 2D and 3D cancer cell cultures. Finally, we showed that these block copolymers could formulate the hydrophobic anticancer drug paclitaxel and induce cytotoxicity *in vitro* comparable to the commercially advanced PTX formulation Genexol PM, while unloaded block copolymers showed no intrinsic cytotoxicity.

In **Chapter 3**, we expanded upon the abovementioned work on different levels. On the monomer level we devised an alternative strategy for the synthesis of carbonate-modified hydroxyl-containing monomers, which allowed us to extent our approach to acrylamides, which were previously inaccessible due to the occurrence of Michael addition as a side reaction. Moreover, besides an ethyl side chain, we explored the introduction of a benzyl side chain to increase micellar stability and drug loading capacity, due to π-π stacking of the aromatic groups in the micelle core. On the level of route of polymerization we elaborated in this work on a controlled radical polymerization technique, more specifically, reversible

addition-fragmentation chain transfer (RAFT) polymerization to obtain polymers with controlled molecular weight, low dispersity and end-group functionality. In this work, efficient RAFT polymerization of both HEAm-EC and HEAm-BC was observed and a library of well-defined block copolymers with different degrees of polymerization (DP) were synthesized. To further explore the versatility of our approach in terms of polymer synthesis, self-assembly, drug solubilization and *in vitro* cell interaction, polyethylene glycol (PEG) and polyHEAm as hydrophilic polymer blocks were compared. The block copolymers formed micellar nanoparticles (10-100 nm) in PBS and could efficiently solubilize hydrophobic dyes and anti-cancer drugs. Benzyl carbonate ester side chains increased micellar stability and drug loading capacity. Moreover, PEG as hydrophilic block showed in comparison to HEAm more promising results concerning both colloidal stability and drug loading capacity. Confocal microscopy showed that the micelles could efficiently deliver a hydrophobic dye inside the cells. Finally, we also demonstrated efficient formulation of the anti-cancer drug paclitaxel with an *in vitro* cancer cell killing performance comparable or even better than Abraxane and Genexol-PM at equal drug dose. Further improvements of this system could be explored in the direction of core crosslinking and covalent drug ligation to ensure stability in the blood stream.

In **Chapter 4**, we elaborated on the concept of active targeting to allow for more selective delivery of drug loaded polymer-based nanomedicines to tissues that overexpress a specific target receptor/antigen. In this work, we explored the use of the high affinity small molecule ligand ACUPA to target prostate-specific membrane antigen (PSMA), which is a membrane receptor that is often overexpressed by prostate cancer cells and neovascular endothelium of solid tumors. In a first step, a fluorescent ACUPA conjugate was synthesized and showed highly efficient targeting of the PSMA-expressing, LNCaP cells, unlike PSMA- negative PC3 cells *in vitro*. This was evidenced by confocal microscopy and FACS. Moreover, co-incubation with the inhibitory ligand 2-(phosphonomethyl)-pentandioic acid (2-PMPA) abrogated ligand binding on PSMA-expressing cells. Encouraged by these findings, we endeavoured into the design of a series of nanomedicines including (1) nanoparticles with ACUPA motifs positioned at the dangling chain ends of a hydrophilic polymer shell, (2) PEGylated lipid nanoparticles with ACUPA motifs at the PEG chain ends and (3) hydrophilic polymers with multiple ACUPA motifs along the polymer backbone. Taken together, we found that although the use of small molecules that contain ACUPA are highly efficient in targeting PSMA-expressing cells, macro-

and supra-molecular structures are much less selective are prone to severe non-specific cellular uptake. This may lead to misinterpretation and we suggest that extreme care should be taken when considering the use of ACUPA as a targeting ligand, in particular with regard to including appropriate controls in the respective studies.

Chapter 5 reports on the combination of the ICD inducer oxaliplatin with the imidazoquinoline-derivative and toll-like receptor (TLR) 7/8 agonist 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinoline-4-amine (IMDQ). We hypothesized that a promising strategy to combine oxaliplatin with IMDQ would be to exploit the Pt (IV) prodrug approach. In particular, the introduction of axial ligands allows to tailor the biological properties by reacting with biologically active ligands, forming multifunctional complexes. IMDQ was coupled via a succinate-spacer to one axial ligand. The remaining axial ligand was modified with a hydrophobic octadecyl (C_{18}) lipid chain to enhance cytotoxicity through increased cellular uptake. The synthesized compound, IMDQ-Pt-C_{18}, was fully characterized by ESI-MS and NMR spectroscopy. CyTOF mass cytometry showed improved cellular uptake of Pt (IV) lipid conjugates compared to unformulated oxaliplatin. *In vitro* cytotoxicity and innate immune activation assays proved that IMDQ-Pt-C_{18} becomes highly active upon cellular uptake. Finally*, in vivo*, we showed that lipid-IMDQ-Pt prodrug allow for site-specific immune activation at the site of administration, without provoking systemic inflammation. Taken together, these initial *in vitro* and *in vivo* results are encouraging to further investigate the therapeutic potential of IMDQ-Pt-C_{18} *in vivo*. Moreover, the rate of reduction of IMDQ-Pt-C_{18} should also be explored to gain further valuable insight in the mechanism of action of this conjugate. Additional efforts should also be devoted to find an optimal pharmaceutical formulation of this compound that properly addresses its limited aqueous solubility.

Chapter 6 positions this work within a broader international context. In a first part, some of the main approved nanomedicines in the clinic are addressed along with the opportunities and relevant challenges that are faced for successful development of nanomedicines. The complexity of tumor targeted delivery of nanocarriers includes (1) the heterogeneity of the EPR-effect, (2) the various barriers hampering active targeting and (3) nanomedicine stability in the bloodstream. Furthermore, important considerations and suggestions for future research are described to improve the clinical translation and therapeutic outcome of cancer

nanomedicine. These include (1) proper patient preselection, so-called personalized nanomedicine, (2) the implementation of noninvasive image-guided companion diagnostics, (3) more-advanced and realistic mouse models and (4) focus on combination therapy. In a second part, the potential of immunogenic cell death (ICD) in cancer therapy is discussed. Although immunotherapies often show exciting clinical responses, they are often limited to only a subset of patients and cancer types. The implementation of immunochemotherapy holds considerable promise to achieve additional or synergistic effects and consequent potent durable therapeutic responses. Therefore, combination therapies of ICD-inducers with certain immunotherapies (adoptive cell transfer (ACT), immune checkpoint blockers (ICBs), toll-like receptor (TLR) agonists and vaccines) have been illustrated. Finally, the future prospects and challenges associated with ICD therapies are provided, which include (1) the identification of optimal doses and administration schedules of ICD inducers, (2) the identification of appropriate biomarker, (3) more reliable *in vitro* biochemical assays and (4) more realistic *in vivo* tumor models.