Summary and general discussion
Chapter 7

Therapeutic proteins in fibrosis

Biologicals, such as antibodies, siRNA, cytokines and enzymes, are becoming increasingly popular in the treatment of a wide range of disorders, as reflected by the rapidly increasing number amongst new therapeutic products\(^1\). In fact, at present well over 100 different protein therapeutics are FDA-approved and it is predicted, based on the large number of therapeutic proteins currently in clinical trials and in clinical use, that these numbers will greatly expand in the coming years\(^1\). Such proteins are promising therapeutic agents, as they display high affinity and specificity for their ligands and additional high tolerability as compared to small-molecule drugs\(^1\).

The most common route of administration for (therapeutic) proteins is an (intravenous) injection. Frequent injections are necessary when the tissue permeability is low and the \(\textit{in vivo}\) half-life is short causing large fluctuations in plasma levels\(^3\). Therefore, this administration route is associated with a high burden to the patient, in particular with chronic indications when long-term treatment is required\(^4\). These disadvantages can be easily avoided by applying a sustained release formulation such as polymeric microspheres, providing the gradual and prolonged release of proteins following a single injection\(^5\).

A prevalent chronic condition of which the progression cannot be fully reversed by therapeutic intervention yet, is fibrosis. This is a condition hallmarked by the dynamic deposition of extracellular matrix (ECM) proteins by myofibroblasts in response to acute or chronic injury, like viral infections or metabolic syndrome\(^6\)\(^-\)\(^8\). In the liver, fibrosis can progress to cirrhosis; a process that may take decades, during which the overall liver architecture is irreversibly distorted, causing high morbidity and mortality in patients worldwide\(^9\). In 2015, liver cirrhosis was ranked in the top 20 of leading causes of death worldwide, accounting for more than 1 million of deaths per year\(^10\). With the increasing prevalence of obesity, as important cause of cirrhosis, it is expected that the number of patients suffering from liver cirrhosis will continue to rise the coming years\(^11\). However, the convincing evidence that fibrosis is a dynamic and, depending on the disease stage, an even reversible process has boosted research to gain insights in the molecular mechanisms involved in fibrosis that could advance to new and effective therapeutic strategies in the near future\(^9\).

Currently, the only available curative treatment option for liver cirrhosis is transplantation surgery. Since viral hepatitis is one of the major causes of cirrhosis, some patients benefit from treatment with a new generation antiviral agent against hepatitis B and C viral infections\(^7\). However, new therapeutic strategies are urgently needed to stop fibrosis progression and even induce fibrosis reversal in all patients suffering from this severe disease\(^7\)\(^,\)\(^9\)\(^,\)\(^12\). Many experimental studies focus on myofibroblasts as the primary target for
antifibrotic therapeutics, as they are the key fibrogenic cells in fibrosis. New treatment strategies should not aim at eradication of these cells, but should reduce their fibrogenic activity and induce ECM-degrading enzymes instead. These cells as primary producers of scar tissue should not only be regarded as the main contributors to fibrosis, but they have a crucial role in maintaining organ integrity in general and can even stimulate resolution of fibrosis by induction of ECM-degrading proteases. There are multiple strategies aiming at reducing the pathogenic function of myofibroblasts, as reviewed by Schuppan et al. This includes manipulation of fibrosis-specific pathways upregulated in these cells, such as procollagen type I or profibrogenic signaling, and using advanced siRNA delivery techniques, like liposomes that passively accumulate in the liver. Another approach is to use ligands that can recognize receptors induced on myofibroblasts in order to target drugs or siRNA.

As summarized in chapter 1, the focus of this thesis is on several therapeutic proteins targeted to the disease-induced platelet-derived growth factor receptor beta (PDGFβR) via this latter approach. We aimed to apply a sustained release formulation for such proteins, in order to develop a patient-friendly formulation providing the gradual and prolonged release of targeted protein therapeutics, thereby assuring a constant plasma drug concentration as desired for chronic treatment.

**Targeting of antifibrotic cytokines**

During fibrosis, cells interact with each other via the release of numerous growth factors and cytokines, which can be either disease-inducing (e.g. the profibrogenic platelet-derived growth factor BB (PDGF-BB) and transforming growth factor beta (TGFβ)) or disease-inhibiting (e.g. the antifibrogenic interferon gamma (IFNγ)). Such disease inhibiting cytokines are very interesting as therapeutic proteins, but as they are notorious for their pleiotropic effects on different cell types, they rarely lead to the desired effects. Targeting could improve their future clinical use. In fact, the clinical use of many biological-based therapeutics in general could be drastically improved using cell-selective delivery. In this way, the therapeutic effectivity is enhanced due to the improved pharmacokinetic and pharmacodynamic profile, while circumventing adverse effects, as reviewed recently.

It is widely known that the PDGFβ-receptor is a disease-induced receptor in the context of fibrosis, highly and specifically expressed on myofibroblasts, which makes it a perfect target for site-specific delivery of antifibrotic compounds. Another extensively studied target highly expressed on activated fibroblasts includes for example mannose 6-phosphate/insulin-like growth factor II receptor, but the sugar-moieties used in this approach are commonly
associated with immunogenicity. Therefore, the PDGFβR was further explored as drug target and a series of compounds all based on the same PDGFβR-targeting moiety were developed in the past decades\textsuperscript{17}: the cyclic PDGFβR-recognizing peptide pPB, that is able to bind to the receptor without activating the downstream signaling cascade\textsuperscript{23}. Chapter 2 describes the rationale behind the PDGFβ-receptor targeting and summarizes the different targeted constructs that were developed in time.

One of the most promising compounds developed in our lab appeared to be the minimalized construct Fibroferon. This construct contains the signaling moiety of the potent antifibrotic cytokine IFNγ, while its extracellular receptor binding part is removed. This so called mimetic IFNγ was targeted to the PDGFβ-receptor by coupling of two pPB-moieties via a PEG linker. In this way, the construct can only interact with and bind to the extracellular PDGFβR, thereby preventing interaction with the widely expressed IFNγR and thus reducing side effects. The improved antifibrotic potential and the reduction of adverse effects of Fibroferon as compared to full length IFNγ were previously shown in mouse models of both acute and chronic liver fibrosis as induced with CCl\textsubscript{4}\textsuperscript{24} and in the UUO model for kidney fibrosis\textsuperscript{25}, respectively. Although antifibrotic effects of Fibroferon were noted and this compound might reach the clinical phase, it remained elusive how this compound worked at the cellular level.

For that reason, in chapter 3, we evaluated the mechanism of action of this chimeric construct by studying the cellular binding, uptake and intracellular signaling and trafficking. We confirmed the binding of Fibroferon to the PDGFβR expressed by myofibroblasts, and interestingly were able to show the absence of activation of the PDGFβ-signaling route. Alternatively, we observed the induction of the signaling cascade of the IFNγ-receptor following clathrin-dependent internalization, and subsequent nuclear translocation and uptake. We showed that after delivery of the signaling moiety of IFNγ to the PDGFβ-receptor we do get IFNγ-associated signaling effects. Therefore, in this study, we demonstrated the possibility of redirecting cytokines to a cellular target of interest in general. Intriguing questions that remained unanswered in this study, concern the switch of Fibroferon from the targeting receptor (PDGFβR) to the effector receptor (IFNγR), escape from the endosomal degradation pathways after uptake via the target receptor, and how the nuclear uptake of such a construct occurs. Moreover, a fascinating question is whether this trafficking is unique for the PDGFβR pathway, or if it is also possible with similar constructs targeting other receptors using other intracellular routes.

Apart from IFNγ, in the past years various studies reported many other cytokines that possess antifibrotic activity as well\textsuperscript{19}. This raised the question whether IFNγ was the absolute best choice for targeting to the main pathogenic cells in fibrosis, or whether there are other even
more promising candidates available. For example, hepatocyte growth factor (HGF) and interferon alpha (IFNα) were reported as antifibrogenic cytokines involved in liver disease\(^\text{19}\). Another example is the adipocytokine adiponectin, which was mainly recognized for its anti-inflammatory properties by inhibiting the synthesis of the proinflammatory tumor necrosis factor alpha (TNFα) and by inducing the two important anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist\(^\text{26}\). However, it can also influence fibrosis in a direct manner by suppressing the proliferation and migration of activated hepatic stellate cells (HSCs) as main myofibroblast-like cells in liver fibrosis. In fact, it was shown by Kamada \emph{et al.} that adiponectin attenuated CCl\(_4\)-induced liver fibrosis in mice\(^\text{27}\).

The aforementioned cytokine, TNFα, and interleukin-1 beta (IL-1β) are commonly known as proinflammatory mediators. Actually, TNFα was shown to contribute to hepatic fibrosis by activating local macrophages to secrete the profibrotic cytokine transforming growth factor beta (TGFβ)\(^\text{19}\). However, a more recent study revealed that both TNFα and IL-1β can also reverse \emph{in vitro} the profibrogenic phenotype of HSCs, by upregulation of several matrix metalloproteinases and downregulation of certain profibrogenic genes (including α-SMA and PDGF-BB)\(^\text{28}\). The targeting of antifibrotic cytokines such as adiponectin and TNFα could possibly be a novel therapeutic strategy in chronic (liver) disease. The essential challenge there is to bring the signaling moiety of the cytokine of interest to the right location inside the cell, \emph{i.e.} cytoplasm or nucleus, following delivery of this moiety to a disease-induced receptor. It remains to be seen whether this exciting strategy is applicable for other cytokines apart from IFNγ. Future research should thus focus on developing other constructs targeting potent cytokines to various disease-induced receptors, in this way creating several new treatment options with fewer side effects for chronic conditions.

In the past, such targeted protein constructs were synthesized chemically in our group, allowing the production of a well-defined product\(^\text{29}\). However, the synthetic upscaling and purification of such constructs sometimes appeared to be problematic in terms of reproducibility, yield and activity of the synthetized protein. Therefore, to explore the applicability of multiple proteins in large \emph{in vivo} studies and eventually clinical trials, an alternative method providing more flexibility in protein design is necessary, such as the biological production of complex molecules or fragments via recombinant techniques\(^\text{30}\). A commonly used host is \emph{Escherichia coli}, which is approved by drug regulatory authorities for recombinant protein production\(^\text{31}\). Inside the host, proper folding into the native conformation is guaranteed by a so-called protein quality control network\(^\text{32}\); a feature that is evidently absent during chemical synthesis of proteins. However, release of the recombinant protein from the cytoplasm of the \emph{E. coli} is accompanied by binding to cellular components including endotoxins (lipopolysaccharides) from which the protein has to be purified before
administration in vivo\textsuperscript{30}. This can be done with affinity chromatography, but this technique is generally associated with low yields of purified protein. An alternative is switching to a different bacterium strain, for example the Gram-positive and thus endotoxin free \textit{Lactococcus lactis}. Although production in \textit{L. lactis} allows easy scale-up for large protein amounts, many proteins are degraded by the internal proteolytic system. However, past and current developments made many advances in improving the protein production and secretion in this alternative host\textsuperscript{32}.

\textbf{Polymeric microspheres for sustained release of therapeutic proteins}

As mentioned earlier in this chapter, polymeric microspheres are a suitable sustained release formulation for therapeutic proteins in the lifelong treatment of chronic diseases like fibrosis, by providing constant plasma concentrations and thus avoiding peak levels. This formulation can be a patient-friendly alternative to repetitive parenteral administrations that are accompanied by high patient discomfort\textsuperscript{5}. Therefore, this formulation can bring the clinical application of such proteins one step closer. Obviously, to date various sustained release formulations are available, like injectable implants\textsuperscript{33} or nanoparticles\textsuperscript{34}. Polymeric microspheres are particularly interesting as they provide stability of the encapsulated therapeutic proteins by physical protection from cells and enzymes in adjacent tissues\textsuperscript{5}. Proteins encapsulated in and released from polymeric microspheres do not have denaturation issues following exposure to high temperatures which is common during the production of implants, generally produced via hot melt extrusion\textsuperscript{35}. Moreover, such polymeric microspheres can be produced relatively easy and their composition can be simply adjusted depending on the preferred release profile and dosage of the protein of interest. Another advantage is that the formulation can be easily administrated via a single subcutaneous injection\textsuperscript{5}.

The most commonly used polymer in this microsphere application is the biodegradable polymer poly (lactic-co-glycolic) acid (PLGA), from which the encapsulated protein is released via degradation of the polymer by bulk erosion\textsuperscript{36}. Currently, there are several protein-delivery systems approved for marketing based on PLGA microspheres. Two examples include Nutropin Depot\textsuperscript{®}, containing recombinant human growth hormone that is indicated for the long-term treatment of growth failure, and Lupron Depot\textsuperscript{®} which contains leuprolide, a synthetic analog of gonadotropin-releasing hormone, indicated for amongst others endometriosis. Such depot products can be administered at 1, 3 or even 6 month intervals\textsuperscript{37}. However, the acidic degradation products and the hydrophobic nature of PLGA can cause (chemical) denaturation or aggregation of encapsulated proteins and this could contribute to
incomplete release and an undesirable release profile\textsuperscript{38}. Alternative biodegradable synthetic polymers for microsphere production include polycaprolactones, or natural polymers such as starch and hyaluronic acid, but unlike PLGA-based microparticles, these polymers never reached clinical approval by the FDA because they displayed lower biocompatibility and biodegradability, or did not possess similar mechanical properties\textsuperscript{35}.

In our studies we used a combination of two biodegradable and biocompatible multi-block co-polymers as matrix of the microspheres. The two different polymers were based on amorphous blocks, composed of poly (ε-caprolactone) (PCL) and poly (ethylene glycol) (PEG), and semi-crystalline blocks, created with poly (L-lactic acid) (PLLA)\textsuperscript{39}. Different to PLGA, the release of proteins from microspheres produced with these polymers is diffusion-mediated instead of degradation-mediated. This diffusion is regulated by the swelling of the PEG fragments in the amorphous blocks when in an aqueous environment following \textit{in vivo} injection\textsuperscript{40}.

We developed a microsphere formulation for PDGFB-receptor directed human serum albumin (pPB-HSA), which is a cell-specific drug carrier\textsuperscript{23}. We extensively studied the \textit{in vitro} release of pPB-HSA from microsphere compositions with different blends of the copolymers in chapter 4, thereby aiming at a 14-day release. Moreover, we conducted an \textit{in vivo} proof of concept study in the UUO mouse model for kidney fibrosis, as the chronic nature of liver fibrosis demands sustained release for a prolonged period of time. We were able to demonstrate gradual and complete \textit{in vitro} release of pPB-HSA within 14 days in the optimized microsphere formulation, with both co-polymers in a 50:50 ratio. Moreover, we showed the \textit{in vivo} release of pPB-HSA in plasma 7 days after a single subcutaneous injection of these microspheres and the enhanced accumulation of the targeted protein in the fibrotic kidney expressing the PDGFB-receptor. In this study, we showed that this microsphere technology is suitable as sustained release formulation for large complex proteins, and can thus be successfully combined with a second technology, \textit{i.e.} drug targeting\textsuperscript{39}.

In chapter 5, we took this concept one step further, by studying the \textit{in vivo} pharmacokinetics of pPB-HSA released from the optimized microsphere formulation in the Mdr2-/- model for liver fibrosis. This study showed that pPB-HSA was released from the subcutaneously residing microspheres. Both the plasma concentration and liver concentrations already reached steady state levels within 1 day after injection and lasted for 5 days. A rather unexpected decline in plasma and liver pPB-HSA levels 7 days after injection was associated with the induction of an antibody response in these mice against the human albumin. This was avoided by substituting human serum albumin with the mouse equivalent, creating the drug carrier pPB-MSA that may be suitable for long-term studies in mice\textsuperscript{41}. Obviously, for clinical studies
and ultimate application in humans the carrier protein should be complementary for use in humans, \textit{i.e.} pPB-HSA. Hence, our results demonstrate a relevant pitfall that can be encountered with exploring the therapeutic potential of biologicals; species differences should always be taken into account, particularly when extrapolating fundamental, experimental studies of protein therapeutics to clinical studies.

We also correlated the \textit{in vitro} release data to our \textit{in vivo} pharmacokinetic data, and estimated a 20-fold lower release rate of pPB-HSA from the microspheres \textit{in vivo}, relative to \textit{in vitro} studies. This lower release rate \textit{in vivo} does not necessarily mean that it will be challenging to obtain therapeutic effects when an active drug is coupled; the antifibrotic effect of the construct greatly depends on the potency of the attached pharmacon. Evidently, the \textit{in vitro} studies are oversimplified as compared to the more complicated situation \textit{in vivo}, in which the anatomy and microenvironment of the subcutaneous space play a key role\textsuperscript{42}, in part explaining the discrepancy between these studies. The \textit{in vivo} plasma levels are the net result of release from the subcutaneous depot, uptake by the target cells and metabolism\textsuperscript{41}.

The essential question that was subsequently raised, was whether the steady state concentrations reached in the target organ (\textit{i.e.} liver) were sufficient to obtain therapeutic levels. In order to find an answer to this question, we attached the potential antifibrotic rho-kinase inhibitor Y27632 to the drug carrier pPB-MSA, encapsulated this in the microsphere formulation and determined the pharmacodynamics \textit{in vivo} in Mdr2-/- mice \textit{chapter 6}. We confirmed that pPB-MSA-Y27632 displayed \textit{in vitro} a relaxing effect on HSCs. After confirming that PDGFβ-receptor directed Y27632 was able to exert its effect in the stellate cells as target cells, we determined its effect \textit{in vivo}, and demonstrated antifibrotic effects as reflected by a reduction in expression of several fibrosis-related genes. Clearly, the concentration of Y27632 in the target cell is high enough to exert its effects, while the untargeted, unencapsulated equivalent does not show any effect following daily subcutaneous injections. However, it would still be interesting to determine the plasma levels of pPB-MSA in the treated mice, to be able to compare these values to the ones found in the pPB-HSA treated animals.

Although the application of the polymeric microspheres used in this thesis was promising, there is still room for improvement. These microspheres were produced by water-in-oil-in-water emulsification by homogenization, resulting in a broad particle size distribution as visible from scanning electron microscopy images and as reflected by high span values. Particle size is an essential property in governing the release profile of the encapsulated therapeutic protein and plays an important role in the fate of particles as well when they might be recognized by (local) macrophages, which can opsonize such non-self objects\textsuperscript{43,44}. By production of the microspheres with a similar double emulsification method via membrane
emulsification, in which the primary emulsion is forced through uniform pores of a glass membrane into the external water phase, a narrow particle size distribution can be obtained\cite{45}. In this way, the release behavior of the encapsulated drug can be controlled more precisely and additionally better biocompatibility can be obtained with cells and tissues in vivo\cite{45}.

**Concluding remarks and new directions**

Liver fibrosis can be caused by viral infections, alcohol and genetic disorders, but obesity leading to non-alcoholic fatty liver disease (NAFLD) including non-alcoholic steatohepatitis (NASH) is nowadays forming one of the major threats to global health\cite{46,47}. Currently, the only available therapeutics for patients with liver fibrosis or cirrhosis are the new generation antiviral agents, but they are only effective in case the disease is caused by hepatitis B and C viral infections\cite{7,9}. Various recent studies suggest that the fibrotic process is halted or even reversed after successful eradication of the causative agent\cite{9}. Several therapeutics are in clinical trials, some of them protein-based, including antibodies for example against the profibrotic cytokine TGFβ1 or the regulator of the ECM-crosslinking enzyme LOXL2\cite{9}. However, most therapeutic proteins to treat liver diseases are not ready yet to enter the market\cite{48}.

Protein-based drugs represent a group of fastest growing new medicines for the treatment of nearly 150 indications, including cancer, diabetes, hepatitis and rheumatoid arthritis. However, clinical application is still limited or associated with severe side effects, frequently related to the administration route, immunogenicity or hepatotoxicity\cite{20}. Sustained release formulations such as the microsphere technology might therefore open patient-friendly therapeutic treatment options for patients suffering from many chronic indications, and such formulations may also enhance the therapeutic efficacy by providing a sustained release\cite{3,49}. In this thesis we were able to show the successful application of the microsphere technology for the delivery of (targeted) therapeutic compounds in the context of (liver) fibrosis.

Clearly, many advances are ongoing in (pre-) clinical drug development for liver diseases, however, there are still many scientific hurdles to take. The focus of future research should be both on the search for the most potent targeted antifibrotic proteins that show the least adverse effects and on a suitable patient-friendly (microsphere) formulation with the right polymer composition. When using the in this thesis described versatile polymer platform, suitable formulations can be designed and developed for any potent proteinaceous antifibrotic drug, possibly derived from endogenous mediators, with any desired release profile. In this way, we will gradually move towards the clinical application of a combined
delivery system that provides the sustained release of effective (targeted) antifibrotic proteins. It is important to unite the forces from both the pharmaceutical technology and drug targeting field, because if you want to go fast, go alone, but if you want to go far, go together (African proverb).
References


