Strategies for Functionalization of Recombinant Spider Silk

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Cover: Illustration of a recombinant spider silk (4RepCT) fiber functionalized with affinity domains and enzyme.

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Abstract

The use of silk-based materials for medical and biotechnological applications has been investigated for many years, with particular progress the last fifteen years. Extensive research has been conducted on silk derived from the silkworm *Bombyx mori*, but lately the evolvement in recombinant production has made mimics of spider silk proteins increasingly available. Revealed characteristics of silk such as biocompatibility, biodegradability and mechanical strength are features highly desirable in materials for medical purpose. This, in combination with techniques for functionalization (addition of new functions), incentivize further development of silk into highly sophisticated materials useful for advanced applications.

The main objective of this thesis has been to investigate novel strategies for functionalization of the recombinant spider silk protein 4RepCT. Two distinct approaches were used, coating and genetic fusion. We showed that coating of silk with polyelectrolytes could be employed to make the fibers electrically conductive as well as fluorescent. Parameters affecting the coating efficiency were investigated, and pH was shown to play an important role.

Genetic engineering was employed to fuse 4RepCT with moderately sized protein domains with inherent binding affinities for IgG, albumin and biotin, respectively. We found that the designed silk fusion proteins could self-assemble into silk-like fibers with preserved affinity of the added domains to bind their intended targets. Moreover, we could also demonstrate a general principle for presentation of biomolecules on IgG-binding spider silk by applying a two-step procedure, exemplified by presentation of VEGF via an anti-VEGF antibody bound to IgG-binding silk. We also showed a proof-of-principle for 4RepCT materials with catalytic activity by genetic linkage of an enzyme.

To investigate potential use of other heterologous hosts for increased production of functionalized silk fusion proteins, the methylotrophic yeast *Pichia pastoris* was evaluated. We demonstrated expression and secretion of an IgG-binding silk fusion protein in *P. pastoris*, although the protein was subjected to degradation as well as glycosylation.

Keywords: spider silk, functionalization, gene fusion, affinity domain, IgG, biomolecule presentation, enzyme immobilization, conductivity, *Pichia pastoris*.

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Dedicated to...

...all silk lovers out there

Där skratt saknas görs få framsteg.

Andrew Carnegie

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Müller C., **Jansson R.**, Elfwing A., Askarieh G., Karlsson R., Hamedi M., Rising A., Johansson J., Inganäs O. and Hedhammar M. (2011). Functionalisation of recombinant spider silk with conjugated polyelectrolytes. *Journal of Materials Chemistry* 21(9), 2909-2915.

II **Jansson R.**, Thatikonda N., Lindberg D., Rising A., Johansson J., Nygren P.-Å. and Hedhammar M. (2014). Recombinant spider silk genetically functionalized with affinity domains. *Biomacromolecules* 15(5), 1696-1706.

III **Jansson R.**, Hin Lau C., Ishida T., Ramström M., Sandgren M. and Hedhammar M. (2015). Expression of a functionalized spider silk protein in the methylotrophic yeast *Pichia pastoris*. *Manuscript*.

IV **Jansson R.**, Courtin C.M., Sandgren M. and Hedhammar M. (2015). Rational design of spider silk materials genetically functionalized with enzymatic activity. *Manuscript*.

Papers I-II are reproduced with the permission of the publishers.

Paper not included in the thesis:

V Müller C., Hamedi M., Karlsson R., **Jansson R.**, Marcilla R., Hedhammar M. and Inganäs O. (2011). Woven electrochemical transistors on silk fibers. *Advanced Materials* 23(7), 898-901.

The contribution of Ronnie Jansson to the papers included in this thesis was as follows:

I Planned, performed and analyzed results for the work involving fiber formation, PEDOT-S staining and mechanical testing. Minor contribution to writing of the manuscript.

II Planned, performed and analyzed results for the work involving three of four silk constructs. Participated in data analysis of the fourth construct. Contributed to writing of the manuscript.

III Planned, performed and analyzed results for the majority of the work involving expression and purification. Participated in interpretation of mass spectrometry results. Major contribution to writing of the manuscript.

IV Planned, performed and analyzed results for the majority of the work. Major contribution to writing of the manuscript.

Abbreviations

A. diadematus	*Araneus diadematus*
ABD	albumin binding domain
ADF	*Araneus diadematus* fibroin
B. mori	*Bombyx mori*
CT	C-terminal (domain)
DMA	differential mechanical analysis
E. australis	*Euprosthenops australis*
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
HFIP	hexafluoroisopropanol
Ig	immunoglobulin
IMAC	immobilized metal ion affinity chromatography
kDa	kilodalton
MaSp	major ampullate spidroin
N. clavipes	*Nephila clavipes*
NHS	N-hydroxysuccinimide
NT	N-terminal (domain)
P. pastoris	*Pichia pastoris*
PEDOT-S	poly(4-(2,3-dihydrothieno[3,4-*b*]-[1,4]dioxin-2-yl-methoxy)-1-butanesulfonic acid, sodium salt)
pI	isoelectric point
PTAA	poly(thiophene acetic acid)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
spidroin	spider fibroin
VEGF	vascular endothelial growth factor
Xyl	endo-1,4-β-xylanase A

1 Introduction

A few years back I came across a review article written by Fiorenzo G. Omenetto and David L. Kaplan entitled “New Opportunities for an Ancient Material” (Omenetto & Kaplan, 2010). During the work of writing this thesis it suddenly struck me that this title is really capturing the progress within the field of silk research. From descriptions in the early eighteenth century on the manufacturing of silk clothing (Bon, 1710) to today's ideas of advanced silk-based materials for use in medicine and biotechnology. There has been an extensive progress in the research of silk materials, particularly in the last forty, or so, years. A major breakthrough in designing advanced silk-based materials, at least in my opinion, is the possibility to introduce new functions to silks. By doing so, functional silk materials useful in a variety of technology fields can be created. In this thesis strategies to design functionalized silk and its applicability will be highlighted.

1.1 Silkworm silk and spider silk

Various types of silks exist in Nature (Sutherland *et al.*, 2012; Sutherland *et al.*, 2010; Guhrs *et al.*, 2000), although the most commonly investigated so far for use in medicine and biotechnology are derived from silkworm silk and spider silk (Schacht & Scheibel, 2014; Kasoju & Bora, 2012; Kluge *et al.*, 2008; Vepari & Kaplan, 2007; Altman *et al.*, 2003). By scanning the literature one can notice that *Bombyx mori* silk are undoubtedly the most frequently studied for such technological applications amongst silkworm silks. This is probably due to that the material is easily obtained in large amounts from *B. mori* cocoons. In contrast, spider silk has traditionally been more troublesome to get hold of in large quantities, although recombinant production technology has introduced other ways to get hold of the material, as will be discussed later. Characteristics of silkworm silk and spider silk such as biocompatibility,

biodegradability and mechanical strength make them to highly suitable candidates for use in medical applications (Vepari & Kaplan, 2007). For those reasons, and the fact that the work presented in this thesis is based on a partial spider silk protein, an introduction to *B. mori* silk and to spider silk will follow.

1.1.1 *Bombyx mori* silk

Silk produced from the silkworm *B. mori* is composed of two types of silk proteins, denoted fibroins, linked together via a disulfide bond (Figure 1). The larger of the fibroins (heavy chain or H-chain) is approximately 350 kDa in size, whereas the smaller fibroin (light chain or L-chain) has a size around 26 kDa (Yamaguchi *et al.*, 1989). In addition, spun fibers are coated with a layer consisting of the glycoprotein sericin (Altman *et al.*, 2003). As the sericin layer has shown immunogenic tendencies (Kurosaki *et al.*, 1999) it has to be removed prior to the use of silk fibroin in medical applications, and this is typically achieved in a degumming process (Rockwood *et al.*, 2011). The silk fibroin is obtained by extraction from harvested *B. mori* cocoons, a procedure that is discussed later.

The primary sequence of the heavy chain fibroin is composed of β-sheet forming (crystallite forming) repeats of Glycine-Alanine/Serine motifs (Zhou *et al.*, 2001). In-between the crystallite regions there are non-repetitive (amorphous) linkers not participating in crystal formation.

1.1.2 Natural spider silk

Spiders can produce different silks with different characteristics depending on the purpose of use. They all have specific functions, for example in vibrational sensing, pray capturing and as safety lines (Winkler & Kaplan, 2000). Up to seven types of silks can be produced in distinct glands by spiders, examples being actiniform, pyriform and major ampullate dragline silk (Hinman *et al.*, 2000). From a mechanical point of view the dragline silk (produced in the major ampulate gland) is the toughest, exhibiting a strength comparable to that of the synthetic polymer Kevlar and exceeding the strength of tendons (Hinman *et al.*, 2000). Due to the mechanical properties, silk proteins used for medical and biotechnological applications are almost exclusively derived from dragline silk.

Spider silk proteins are generally referred to as spidroins (spider fibroin). Dragline silk is composed of two spidroins, major ampullate spidroin 1 (MaSp1) and MaSp2 (Humenik *et al.*, 2011), although these are called *Araneus diadematus* fibroin 3 (ADF3) and ADF4, respectively, for the spider *A. diadematus*. The protein architecture of MaSp (Figure 1) is composed of a tripartite structure with a highly repetitive sequence flanked by non-repetitive

N-terminal and C-terminal domains (Rising *et al.*, 2006; Motriuk-Smith *et al.*, 2005; Sponner *et al.*, 2005; Xu & Lewis, 1990). The MaSp architecture (Figure 1) found for the repetitive sequence is similar among dragline silks, with two alternating repeat units. One of the repeat unit, $(Alanine)_n$, provides the fiber strength by forming β-sheets and the other, Glycine-rich unit, provides elasticity (the so-called amorphous region) (Gatesy *et al.*, 2001; Simmons *et al.*, 1996). The non-repetitive N-terminal and C-terminal domains have shown importance in storage and assembly of spidroins (Askarieh *et al.*, 2010; Hagn *et al.*, 2010).

Spidroins are stored in the spiders' glands as a liquid solution (dope) at protein concentrations up to 50% (w/v) (Hijirida *et al.*, 1996). The conformation of the proteins in the dope is mainly α-helical or disordered (Dicko *et al.*, 2004; Hijirida *et al.*, 1996). During the passage through the spinning duct the dope is exerted to changes in composition of ions, decrease in pH [mediated by carbonic anhydrase (Andersson *et al.*, 2014)], shear forces and water removal. These changes induce a conversion of the spidroin dope into a β-rich solid fiber (Romer & Scheibel, 2008; Dicko *et al.*, 2004; Vollrath & Knight, 2001).

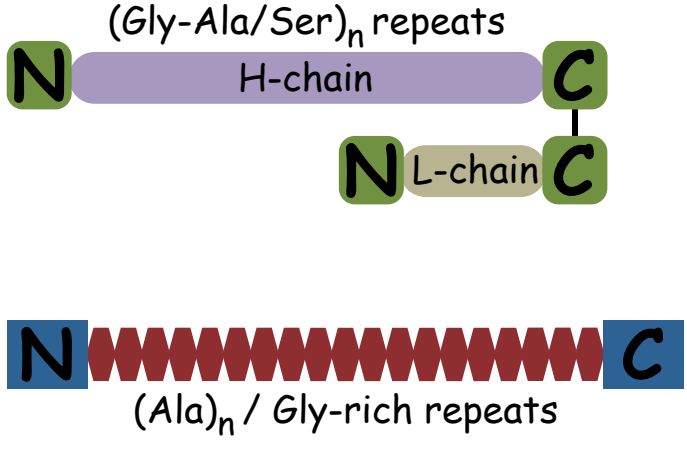

Figure 1. Schematics showing the general architecture of fibroins from the silkworm silk of *B. mori* (upper panel) and spidroins from spider dragline silk (lower panel). *B. mori* silkworm silk is composed of two types of proteins called fibroins. The larger fibroin, heavy chain (H-chain), and the smaller, light chain (L-chain), are linked together by a disulfide bond. The sequence of the heavy chain is rich in glycine and consists of $(Glycine\text{-}Alanine/Serine)_n$ repeats. Spider silk proteins, spidroins, from dragline silk are mainly composed of alternating poly-Alanine and Glycine-rich repeats flanked by non-repetitive terminal domains. N, C = non-repetitive N- and C-terminal domain, respectively.

1.1.3 Recombinant spider silk

By using DNA technology synthetic spidroin genes have been designed for production in heterologous hosts. (Expression of spidroin fragments and mimetics in various host systems is discussed in next chapter.) Sequence repeats important for the transition into β-sheet rich silk structures can be genetically designed and incorporated into an expression vector, followed by expression in a suitable host. The reported designed repeat sequences have been derived from, for example, MaSp1 and MaSp2 from *Nephila clavipes*, ADF3 and ADF4 from *A. diadematus* and MaSp1 from *Euprosthenops australis*, with or without non-repetitive domains included (Askarieh *et al.*, 2010; Stark *et al.*, 2007; Huemmerich *et al.*, 2006; Huemmerich *et al.*, 2004; Mello *et al.*, 2004; Arcidiacono *et al.*, 2002; Fahnestock & Irwin, 1997).

1.2 Recombinant production of spider silk proteins

The territorial and cannibalistic behavior of spiders makes them difficult to farm (Scheibel, 2004). In addition, the low amount of produced silk that can be harvested directly from spiders is not satisfying (Chung *et al.*, 2012), especially not from an industrial point of view. Therefore, recombinant production is considered as the ultimate route forward in producing spider silk proteins in large scale (Tokareva *et al.*, 2013; Rising *et al.*, 2011). Over the years various host systems have been employed, including both prokaryotic and eukaryotic organisms, although the bacterium *E. coli* is by far the most exploited host.

1.2.1 Production in *Escherichia coli*

Due to ease of genetic manipulation, short doubling time and low-cost production, *E. coli* has been widely used for recombinant expression of various spidroin fragments. Frequently reported is expression of spidroin fragments originating from the golden orb weaver spider *N. clavipes* and from the garden cross spider *A. diadematus*. Examples of expressed partial spidroins from *N. clavipes* are repeats from MaSp1 and MaSp2, glycine-rich repeats and the natural C-terminal part (Mello *et al.*, 2004; Arcidiacono *et al.*, 2002; Arcidiacono *et al.*, 1998; Fukushima, 1998; Fahnestock & Irwin, 1997; Prince *et al.*, 1995). Likewise, repeats from ADF3 and ADF4 have also been expressed using *E. coli* as host (Liebmann *et al.*, 2008; Metwalli *et al.*, 2007; Huemmerich *et al.*, 2006; Huemmerich *et al.*, 2004). Moreover, a partial spidroin, 4RepCT, derived from MaSp1 of *E. australis* dragline silk was also expressed in *E. coli* (Stark *et al.*, 2007). The 4RepCT module consists of four poly-alanine/glycine-rich repeats, as well as the native C-terminal domain. The work presented in this thesis is based on the partial spidroin 4RepCT.

Recombinant expression of partial spider silk proteins in *E. coli* is, however, not entirely free from complications. The general architecture of spidroin genes, containing GC-rich stretches, can cause problems with stable mRNA structures which, in turn, lead to ribosome stalling or premature translation termination (Arcidiacono *et al.*, 1998; Fahnestock & Irwin, 1997; Prince *et al.*, 1995). Depletion of tRNA pools due to the abundance of certain amino acids in spidroin sequences may also occur for expression in *E. coli*, as opposed to the situation in the spider's silk glands (Candelas *et al.*, 1990). Furthermore, the large transcript sizes of native dragline spidroins, approximately 10 000 base pairs (Ayoub *et al.*, 2007), are complicating cloning and are probably not well-suited for efficient expression in *E. coli*. This is the main reason for shorter (partial) spidroin fragments being expressed instead. However, one study has shown upon successful expression of a recombinant spidroin of native size (285 kDa) in engineered *E. coli* (Xia *et al.*, 2010). Low solubility of expressed spider silk proteins (Bini *et al.*, 2006; Winkler *et al.*, 2000) is of general concern, but improved solubility has been achieved, for example, by fusion of a solubility-enhancing partner to the partial spidroin 4RepCT (Stark *et al.*, 2007).

1.2.2 Production in *Pichia pastoris*

The yeast *Pichia pastoris* (*P. pastoris*) has attracted great attention as a eukaryotic host for recombinant protein production for a variety of reasons (Ahmad *et al.*, 2014; Daly & Hearn, 2005). The possibility to grow *P. pastoris* to high cell densities prior to induction of protein expression allows high intracellular titers of produced protein to be obtained (Cereghino & Cregg, 2000; Hasslacher *et al.*, 1997). In addition, due to the eukaryotic machinery for post-translational modifications, proteins produced in *P. pastoris* can be modified with, for example, glycosylation and the formation of disulfide bonds can be promoted (Trimble *et al.*, 2004; Bretthauer & Castellino, 1999; Duman *et al.*, 1998; White *et al.*, 1994). The correct function of many eukaryotic proteins is dependent upon post-translational modifications (Kukuruzinska & Lennon, 1998). However, considering glycosylation, the glycan pattern added by *P. pastoris* is different from that in mammalian organisms, which can be problematic if the protein is going to be used in pharmaceutical applications (Daly & Hearn, 2005).

The methanol-inducible alcohol oxidase promoter allows for strong and tight regulation of protein expression in methylotrophic *P. pastoris* (Tschopp *et al.*, 1987; Cregg *et al.*, 1985). Expressed proteins can be secreted into the extracellular growth medium directed by, for example, the α-mating-factor pre-pro leader sequence from *Saccharomyces cerevisiae*. The signal peptide is

subsequently cleaved from the target protein upon secretion (Waters *et al.*, 1988; Brake *et al.*, 1984; Julius *et al.*, 1984). The ability of protein secretion greatly facilitates downstream purification steps, which also lowers the production costs (Fahnestock *et al.*, 2000).

Intracellular expression in methylotrophic *P. pastoris* of spider dragline silk multimers (up to 3000 codons in length) derived from gene analogs of *N. clavipes* MaSp1 has been reported previously (Fahnestock & Bedzyk, 1997). The problem with premature translational termination (*i.e.*, protein truncation) was not observed, in contrast to expression of the same silk genes in *E. coli* as reported in Fahnestock & Irwing, 1997. However, expression of genes longer than 1600 codons was less efficient than expression of shorter ones. Furthermore, a range of various sizes of expressed proteins was evident, and postulated to be caused by recombination events during integration of the silk gene analogs into the *P. pastoris* genome. Produced silk proteins were readily soluble for expression times up to 48 h, however, proteins expressed for longer times appeared in insoluble fraction and were only soluble in 6 M guanidine-HCl. In another study, two silk protein analogs from *N. clavipes* and *N. madagascariensis*, respectively, were expressed intracellular in methylotrophic *P. pastoris* using methanol induction (Bogush *et al.*, 2009).

An amphiphilic silk-like protein has been expressed in *P. pastoris* using fed-batch fermentation and secreted into the growth medium at 1 g L^{-1} (Werten *et al.*, 2008). Secreted silk-like proteins formed insoluble complexes with *P. pastoris*-derived proteins, although target proteins could be effectively purified by solubilization of the complexes in formic acid, followed by precipitation of contaminants by dilution with water. Fed-batch fermentation using *P. pastoris* has also been used to produce and secrete silk-collagen-like proteins at yields of 1-3 g L^{-1} growth medium after protein purification by selective precipitation (Golinska *et al.*, 2014; Wlodarczyk-Biegun *et al.*, 2014).

1.2.3 Production in other hosts

Other hosts have, indeed, also been utilized for recombinant production of spidroin fragments. Production in plants, mammalian cells and transgenic animals has been reported over the years.

Transgenic tobacco, potato and *Arabidopsis thaliana* plants have been used for expressing repeats from MaSp1 and MaSp2 of *N. clavipes* (Yang *et al.*, 2005; Menassa *et al.*, 2004; Scheller *et al.*, 2001). For expression using mammalian cell culture, bovine mammary epithelial alveolar cells immortalized with large T (MAC-T) and baby hamster kidney cells was investigated for the suitability to express spider dragline silk analogs (Lazaris *et al.*, 2002). Sequences coding for fragments of MaSp1 and MaSp2 from *N.*

clavipes and of ADF3 from *A. diadematus* were expressed, and recombinant ADF3 silk protein was obtained at 25-50 mg L^{-1}. Secretion of a sequence combining parts from MaSp1 and MaSp2 from *N. clavipes* into milk was achieved using transgentic mice, although the maximum concentration of obtained silk proteins was 12 mg L^{-1} (Xu *et al.*, 2007). Moreover, a dragline silk fragment of MaSp1 from *N. clavata* was produced in cocoons of transgenic *B. mori* (Wen *et al.*, 2010). In general, long development times and low amounts of produced silk proteins is so far a limiting factor for production in other hosts.

1.3 Formulation of silk materials

Before giving examples on various silk formats, a condensed note on different ways to obtain pure silk proteins will be given. Different routes for obtaining pure partial spider silk proteins after recombinant expression have been employed, and two examples will now be given. One reported way of purifying partial ADF3 and ADF4 spider silk proteins involves precipitation of *E. coli* proteins from cell lysate by heat denaturation (70-80°C), a step in which the spider silk proteins remain soluble. Next, soluble silk proteins are precipitated by 20-30% ammonium sulfate and harvested by centrifugation. Recovered pellets of silk protein are dissolved in 6 M guanidinium chloride or guanidinium thiocyanate, followed by dialysis and subsequent lyophilization (Huemmerich *et al.*, 2004). Another approach, which is the one that has been employed in this thesis for the partial spidroin 4RepCT, is to use IMAC purification of cell lysate, utilizing a hexa-histidine tag incorporated into the 4RepCT sequence. Bound silk proteins are eluted from the IMAC column using a physiological-like Tris-HCl buffer (pH 8), and subsequently dialyzed against Tris-HCl buffer (Stark *et al.*, 2007).

As silk materials made from both spider silk and silk fibroin (Figure 1) will be dealt with in parallel throughout the rest of this thesis, a short note on how to obtain silk fibroin will follow. Silk fibroin is extracted from raw silk harvested from, for example, *B. mori* cocoons. The sericin coat, adhesive glycoproteins covering the surface of fibroin fibers, can for example be removed by boiling the cocoons in 0.02 M sodium carbonate, and the silk is thereby said to be degummed. Soluble regenerated silk fibroin can then be obtained by for example immersing the degummed silk in 9.3 M lithium bromide, followed by dialysis against water and optional lyophilization (Rockwood *et al.*, 2011).

Various silk materials have been formulated from solutions of purified partial spider silk protein, as well as from regenerated silk fibroin, most

commonly derived from *B. mori* cocoons. Silk films (Gil *et al.*, 2010; Widhe *et al.*, 2010; Lawrence *et al.*, 2009; Huang *et al.*, 2007; Metwalli *et al.*, 2007; Bini *et al.*, 2006; Foo *et al.*, 2006; Huemmerich *et al.*, 2006; Junghans *et al.*, 2006; Slotta *et al.*, 2006; Jin *et al.*, 2004; Scheller *et al.*, 2004; Szela *et al.*, 2000; Valluzzi *et al.*, 1999; Winkler *et al.*, 1999; Fukushima, 1998) and fibers (Wharram *et al.*, 2010; Ghosh *et al.*, 2009; Rammensee *et al.*, 2008; Exler *et al.*, 2007; Stark *et al.*, 2007; Foo *et al.*, 2006; Li *et al.*, 2006; Stephens *et al.*, 2005; Arcidiacono *et al.*, 2002) are the two most investigated materials, although formulation into silk hydrogels (Etienne *et al.*, 2009; Yucel *et al.*, 2009; Rammensee *et al.*, 2006), microspheres (Lammel *et al.*, 2008; Slotta *et al.*, 2008; Wang *et al.*, 2007) and microcapsules (Li *et al.*, 2014; Hermanson *et al.*, 2007) are also reported.

1.3.1 Films

Films casted from purified recombinant spider silk proteins have been widely investigated, probably because of the ease of preparation. Two spider silk proteins $(AQ)_{12}$ and C_{16}, being sequence repeats derived from ADF3 and ADF4 of *A. diadematus*, respectively, were casted from purified protein dissolved in 100% hexafluoroisopropanol (HFIP) on a polystyrene surface (Slotta *et al.*, 2006). After evaporation of HFIP all films could be solubilized in water. Interestingly, if the films instead were treated with 100% methanol or with 1 M potassium phosphate (pH 8.0) they became more stable as evaluated by the water-insolubility properties. Moreover, FTIR analysis of films after treatment revealed a conversion from α-helical rich to β-sheet rich secondary structure as compared to non-treated films, which could explain the water-insolubility after film treatment. Rendering casted films water-insoluble by post-treatment with alcohols has also been employed for casting of recombinant spider silk films in other studies (Wohlrab *et al.*, 2012; Spiess *et al.*, 2011; Spiess *et al.*, 2010; Huemmerich *et al.*, 2006). There has also been reported on recombinant spider silk films casted from physiological-like buffer that were water-insoluble without further post-treatment, possessing a predominant β-sheet structure directly after drying (Widhe *et al.*, 2013). These films were casted from the partial spider silk protein 4RepCT in solution, and the same methodology for preparation of films were used for the work presented in this thesis.

From secondary structure analysis of regenerated *B. mori* silk fibroin solutions it was determined that the use of methanol increased the β-sheet content in the fibroin molecules, *e.g.* (Canetti *et al.*, 1989), therefore methanol can also be used to stabilize films made from silk fibroin (Rockwood *et al.*, 2011; Jin *et al.*, 2004). To produce films with increased flexibility, mixing of

soluble silk fibroin with plasticizers, like glycerol, prior to film casting has been successfully tried (Lu *et al.*, 2010). It was envisioned that the increased film flexibility could be a beneficial feature in biomaterial and device applications.

Other techniques than direct casting of silk films have also been adopted. Dip and spin coating were used to generate thin films (coatings) from recombinant spider silk proteins (Zeplin *et al.*, 2014; Junghans *et al.*, 2006). Further on, it was shown that shear forces during spin coating could help native silk fibroin proteins obtained from *B. mori* silkworm glands to self-assemble (Greving *et al.*, 2012).

1.3.2 Fibers

Different techniques have been used to convert soluble spider silk proteins into solid fiber-like structures, mostly of nm-µm scale in diameter. One of the reported method is wet spinning (Bogush et al., 2009; Brooks et al., 2008; Teule et al., 2007; Arcidiacono et al., 2002; Lazaris et al., 2002). The concept for wet spinning of silk (Liivak et al., 1998) partly tries to mimic the natural fiber assembly process in spiders by allowing a highly concentrated silk protein solution to be extruded through a narrow orifice, usually through a needle connected to a pump. Extruded silk proteins are immediately entered into a dehydrating coagulation bath (*e.g.*, methanol), after which collected fibers can be treated by drawing or pulling to further improve the mechanical properties by molecular alignment (Carmichael & Viney, 1999; Kerkam *et al.*, 1991). Spinning of fibers from a silk protein derived from MaSp2 of *Argiope aurantia* resulted in fibers with impaired mechanical properties compared to corresponding native silk fibers (Brooks *et al.*, 2008). This discrepancy was suggested to partly come from the smaller sizes of silk proteins used, compared to the native proteins, and a less efficient spinning procedure than found in spiders.

Another technique that has often been used for production of spider silk fibers is electrospinning (Bogush *et al.*, 2009; Bini *et al.*, 2006; Foo *et al.*, 2006; Stephens *et al.*, 2005). In the technique of electrospinning a polymer solution is forced through a thin nozzle, also serving as an electrode. A high electric field is applied between the electrode and a counter electrode, and on its way between the two electrodes the solvent of the polymer solution evaporates and solid polymeric material can be collected at the counter electrode (Greiner & Wendorff, 2007). A spider dragline silk analog was electrospun from HFIP and the secondary structure content in prepared fibers was compared to that of casted films (Stephens *et al.*, 2005). A predominant β-sheet structure was observed in casted films, whereas a majority of α-helical

structure was found in the electrospun fibers. The conclusion was made that protein conformation can be affected by the electrospinning process itself. Electrospinning has also been widely used in formation of fibers from silk fibroin (Zhang *et al.*, 2012; Wharram *et al.*, 2010; Ghosh *et al.*, 2009; Li *et al.*, 2006).

Self-assembly of silk fibers from a solution of partial spidroins is yet another approach that has been reported (Stark *et al.*, 2007) and also the method used in this thesis. Partial spidroins derived from MaSp1 of *E. australis*, including its native C-terminal part, spontaneously self-assemble into meter-long fibers in a non-denaturing buffer. The measured mechanical strength of self-assembled fibers was in the range of tendons, although it was weaker than artificially reeled *Euprosthenops* dragline silk. Finally, it can be mentioned that production of spider silk fibers also has been attempted using hand-drawing (Exler *et al.*, 2007; Teule *et al.*, 2007) and microfluidics (Renberg *et al.*, 2014; Rammensee *et al.*, 2008).

1.3.3 Hydrogels

Hydrogels are polymeric structures that absorb water to a high degree, and hydrogels prepared both from recombinant spider silk proteins and from silk fibroin have been accomplished. A protein analog (C_{16}) to the dragline silk protein ADF4 was used to prepare a spider silk hydrogel with viscoelastic properties (Rammensee *et al.*, 2006). The hydrogel was prepared by dissolving lyophilized silk protein in 6 M guanidinium thiocyanate prior to dialysis against 5 mM potassium phosphate (pH 8.0). Upon addition of 10% (v/v) methanol the silk protein self-assembled into nanofibers that subsequently formed a hydrogel fiber network. In a later study, a more controlled hydrogel formation process of the C16 protein with adjustable pore sizes from 10-200 μm was presented (Schacht & Scheibel, 2011). Preparation of hydrogels from silk fibroin have also been achieved, were silk gelation was induced either by sonication followed by incubation under controlled temperature (Etienne *et al.*, 2009) or by vortexing (Yucel *et al.*, 2009).

1.3.4 Microspheres

Bead-shaped morphologies consisting of silk throughout are commonly referred to as silk microspheres. The engineered spider silk protein eADF4(C16) was used to produce silk microspheres through a salting-out process, in which potassium phosphate (pH 8) at concentrations above 400 mM was added to the silk protein solution with applied mixing (Lammel et al., 2008; Slotta et al., 2008). Generated microspheres were rich in β-structure and with the sphere size controllable by the parameters of protein concentration and

mixing intensity. Regenerated silk fibroin has also been processed into microspheres in sizes of approximately 1 μm in diameter by addition of methanol or ethanol to a silk fibroin solution prior to freeze drying (Nam & Park, 2001). Other processes to formulate silk fibroin microspheres include the use of lipid vehicles as template for generating β-sheet rich silk microspheres (Wang *et al.*, 2007) and spray-draying of fibroin solution in combination with exposure to high humidity (Hino *et al.*, 2003).

1.3.5 Microcapsules

The term silk microcapsule refers to a particle with a liquid or hollow core surrounded by a silk shell. Spider silk microcapsules were produced by emulsifying an aqueous ADF4(C16) spider silk solution in toluene (Hermanson *et al.*, 2007). In this way, silk microcapsules rich in β-structure self-assembled into a nanometer-thin film at the toluene/water interface. Using silk fibroin, hollow silk microcapsules were produced by a layer-by-layer coating of silk onto the surface of polystyrene template particles (Li *et al.*, 2014). After removal of the polystyrene template, hollow silk capsules were obtained.

1.3.6 Foams

Three-dimensional foam structures have been manufactured from both recombinant spider silk proteins (Widhe *et al.*, 2010; Agapov *et al.*, 2009) and from silk fibroin (Hofmann *et al.*, 2007; Kim *et al.*, 2005). By salt leaching, lyophilized recombinant spidroin dissolved in 10% lithium chloride and 90% formic acid was converted into three-dimensional scaffolds with partly interconnected pore cavities (Agapov *et al.*, 2009). In another study the partial spidroin 4RepCT contained in a physiological buffer was used for foaming by introduction of air into the spidroin solution by pipetting (Widhe *et al.*, 2010). This procedure for creating foam structures has also been utilized in this thesis.

Three-dimensional structures have also been prepared from regenerated silk fibroin solutions using, for example, granules as template for pore formation (Kim *et al.*, 2005; Nazarov *et al.*, 2004).

1.4 Functionalization of silk

The concept of adding new functions, or features, to silk is generally referred to as *functionalization* of silk. Equipping silk with additional functions is considered as a valuable route forward in producing highly sophisticated silk materials with applicability in medicine and biotechnology (Schacht & Scheibel, 2014; Tao *et al.*, 2012b; Rising *et al.*, 2011; Zhang *et al.*, 2009). In order to realize functionalized silk materials from both recombinantly produced

spider silk proteins and from regenerated silk fibroin various strategies have been employed (Humenik *et al.*, 2011; Hardy & Scheibel, 2010; Murphy & Kaplan, 2009). The main choice to be made prior to silk functionalization is whether to use a non-covalent or a covalent approach. Non-covalent functionalization techniques rely upon non-covalent chemical interactions (*e.g.*, attractive electrostatic interactions) as in the method of adsorption (coating). However, physical capture (entrapment) can also be used as in encapsulation. In contrast, covalent functionalization is based on covalent (chemical) coupling between the material and the functionalizing molecule. An advantage of covalent coupling is the establishment of a stable bond over time. The covalent approach can be accomplished by targeting reactive amino acids in the silk protein, or by genetic fusion on the DNA level that will result in a covalent amide bond upon protein translation of the fusion proteins.

Some examples of the different strategies employed for silk functionalization will be presented below. Extensive work has been conducted on silk fibroin, but herein the focus will be on what has been done with spider silk for the given examples.

1.4.1 Non-covalent: Adsorption

The technique of adsorption, or coating, relies on diffusion of coating molecules to the silk material, whereupon attraction through non-covalent chemical interactions is established between the coating molecules and the silk. Single filaments of natural dragline spider silk from *Araneus ventrocosus* were rendered fluorescent by coating with alternating layers of polyelectrolyte (PE) and cadmium telluride (CdTe) nanocrystals (Chu & Sun, 2007). By immersion of silk filaments in alkaline solution (pH 9.0) the negatively charged silk could bind positively charged PE which, in turn, attracted negatively charged CdTe nanocrystals. Deposited layers onto silk fibers in the form of PE/CdTe/PE generated fibers emitting bright fluorescence, with the mechanical properties of coated fibers not significantly changed.

Adsorption was used to dip-coat collected dragline silk fibers (*A. ventrocosus*) with multi-walled carbon nanotubes for induction of electrical fiber properties (Kim *et al.*, 2007). The surface of fibers was coated with carbon nanotubes as visualized by scanning electron microscopy (SEM) and an electrical conductivity of $6 \cdot 10^{-3}$ S cm^{-1} was measured. Although the exact coating mechanism was not elucidated, it was speculated that the non-ionic detergent Triton X-100, used to disperse the carbon nanotubes, may act as a link and provide hydrophobic interactions between the silk fiber and the carbon nanotubes. In another study, carbon nanotubes were uniformly coated onto harvested dragline silk fibers (*N. clavipes*), probably promoted by a

combination of ionic interactions and hydrogen bonding (Steven *et al.*, 2013). Coated fibers displayed an electrical conductivity of 12-15 S cm^{-1}. Fibers from natural dragline spider silk (*Nephila edulis*) has also been prepared to gain magnetic properties by coating with a magnetite nanocrystal layer from a Fe_3O_4 solution (Mayes *et al.*, 1998). Mechanical testing of magnetite-silk fibers revealed negligible difference in mechanical properties from the coating.

A homogeneous layer of crystalline calcium phosphate (CaP) was coated onto silk fibers made from the recombinant spidroin 4RepCT (Yang *et al.*, 2010). In the biomimetic coating process fibers were immersed into a stimulated body fluid solution at near-physiological conditions, and it was determined that the coating started with deposition of sodium chloride crystals prior to adsorption of the CaP layer. Another example is the use of electrostatic attraction to adsorb small molecules to silk microspheres produced from the recombinant spidroin eADF4(C16) (Lammel *et al.*, 2011). Small molecular model drugs were mixed with silk microspheres in solution at pH 6.5, at which the microspheres are negatively charged due to the nature of eADF4(C16). Analysis of loading efficiency revealed that small molecules with permanent positive charges were efficiently incorporated into the microspheres, whereas molecules with negative charges could not be incorporated. It was concluded that the small molecules initially were attracted to the surface of silk microspheres by electrostatic forces, after which they diffused into the spheres and were bound by attractive electrostatic and hydrophobic interactions. In the presented concept of using silk microspheres as drug carriers, release of the adsorbed molecules could also be shown. In a model system for silk particles loaded with protein drugs, spider silk microspheres composed of eADF4(C16) were investigated for adsorption of a 14 kDa lysozyme protein (Hofer *et al.*, 2012). Successful microsphere loading of up to 30% (w/w) lysozyme due to attractive electrostatic interactions was achieved. The lysozyme was not only adsorbed to the surface of the microspheres, but also diffused into the protein matrix.

1.4.2 Non-covalent: Co-precipitation

A different technique for incorporation of molecular ingredients into spider silk materials is co-precipitation. In this method spider silk proteins in solution are mixed with the molecule ingredient to be incorporated, after which the silk/molecule ingredient solution is precipitated. Upon precipitation, silk microspheres are formed with the molecule ingredient incorporated into the spheres.

An example of applying the co-precipitation technique is the incorporation of β-carotene into ADF4(C16) spider silk microspheres (Liebmann *et al.*,

2008). A solution blend consisting of spider silk protein and β-carotene was used to initiate co-precipitation and microsphere formation by addition of at least 600 mM potassium phosphate (pH 8). Due to the strong hydrophobic nature of β-carotene it easily aggregates in aqueous solution and therefore, is likely to concentrate in the hydrophobic silk phase created in the microsphere formation process. Incorporation of β-carotene in the silk microspheres (1-5 μm in diameter) was observed with a maximum loading of 5% (w/w). Release of incorporated β-carotene was also demonstrated by active degradation of the microspheres using Proteinase K. The silk microsphere co-precipitation approach was envisioned as an alternative in overcoming problems related to formulation and delivery for poorly water-soluble substances. Co-precipitation was also examined for formulation of eADF4(C16) silk microspheres using the fluorescent dye rhodamine B as a drug model (Blum & Scheibel, 2012). Loading of rhodamine B by co-precipitation was more effective than loading of microspheres by diffusion, and it was suggested that diffusion loading may only lead to surface bound rhodamine B.

1.4.3 Non-covalent: Encapsulation

The use of silk microcapsules for enclosure of water-soluble molecules was demonstrated using capsules made from the spider silk protein C16, derived from ADF4 (Hermanson *et al.*, 2007). Dextran (40 kDa) labeled with fluorescein isothiocyanate (FITC) was solubilized in the silk protein solution, after which emulsification of the silk/FITC-dextran suspension was achieved using toluene. Silk proteins were adsorbed to the surface of the generated water-based droplets, thereby encapsulating the FITC-dextran molecules, as visualized by fluorescence microscopy. The silk membrane of microcapsules was impermeable to large dextrans, whereas low molecular-weight FITC molecules could freely diffuse across the membrane. Moreover, release of microcapsule content could be enzymatically triggered by degradation of the silk shell using Proteinase K. It was speculated that any small molecule could be encapsulated using silk microcapsules as long as the small molecule do not affect the assembly of the silk shell, or is itself affected by the encapsulation process (Hermanson *et al.*, 2007).

Silk microcapsules for encapsulation of protein molecules was shown using β-galactosidase as a model enzyme (Blum *et al.*, 2014). To avoid the toxic effect from toluene previously used in the emulsification process, non-toxic medical grade silicon oil was used instead. Microcapsules with a shell composed of eADF4(C16) silk proteins and enzymes trapped inside were produced. However, the use of mineral oil for emulsification required an additional immersion of the capsules in ethanol to induce β-sheet formation in

the silk shell. Encapsulated enzymes were catalytically active as shown by their ability to convert colorless substrate into colored products. Furthermore, on-demand activation of an encapsulated inactive variant of β-galactosidase was demonstrated by allowing an activation peptide to diffuse through the silk membrane and subsequently activating the enzyme. Protection from proteolysis with endoproteinase AspN was shown for encapsulated enzymes.

1.4.4 Covalent: Chemical coupling

Functionalization of spider silk materials can be achieved by chemical modification of reactive amino acids [*e.g.*, lysine, tyrosine, glutamic acid and aspartic acid (Basle *et al.*, 2010; Thordarson *et al.*, 2006)] in the silk protein sequence. Such chemical modifications can introduce covalent bonds, thereby allowing more stable coupling of additional molecules to the spider silk.

To show upon the principle of spider silk functionalization by covalent coupling, silk films were prepared from the partial spidroin C16 and subsequently treated for binding to the fluorescent dye FITC and to the enzyme β-galactosidase (Huemmerich *et al.*, 2006). For covalent coupling of the small organic FITC molecules and the larger macromolecular enzymes, respectively, a carboxyl-to-amine linkage was created between silk proteins in the films and FITC/enzyme (via an ethylenediamine linker in the case of FITC). This was achieved by activation of available carboxyl groups on the film surface and subsequent binding to primary amines in the FITC-conjugated linker or in the enzymes mediated by a so-called EDC/NHS reaction (EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, N-hydroxysuccinimide). Coupling of both types of molecules to silk films was successful, as visualized by emitted light from silk/FITC films and accumulation of colored product for silk/enzyme films, respectively. In a similar manner, spider silk hydrogels were prepared from spidroin C16 proteins covalently coupled to fluorescein molecules (Schacht & Scheibel, 2011). Covalent coupling was achieved using 5(6)-carboxyfluorescein N-hydroxysuccinimid ester to target the amino-terminus of spidroins, although it was established that the binding was not restricted to the amino-terminus as multiple bound fluorescein molecules per silk molecule was detected. Hydrogel formation of silk-fluorescein proteins was observed, although the gel-forming kinetics and mechanical strength were impaired compared to non-functionalized silk hydrogel. It was suggested that the bound fluorescein molecules interfere with the molecular packing of silk molecules, consequently altering assembly kinetics and mechanical properties of the silk-fluorescein hydrogel.

As a proof of site-specific covalent modification of spider silk materials the amino acid cysteine has been targeted for coupling in engineered spider silk

proteins (Spiess *et al.*, 2010). Modified variants of the partial spidroin eADF4(C16) were genetically engineered on the DNA level to contain a cysteine residue either at the amino-terminus or carboxy-terminus, resulting in Cys-C16 spidroins. No structural differences of the Cys-C16 variants in solution or when processed into films were observed compared to the non-modified C16 spidroin. To show covalent binding to the incorporated cysteine residue, fluorescein-5-maleimide was bound to reduced thiol groups in Cys-C16 in soluble form. Covalent linkage of fluorescein to Cys-C16 in solution was confirmed, as was the site-specificity of the binding. One Cys-C16 variant with bound fluorescein was also effectively casted into film. Moreover, thiol groups in already assembled Cys-C16 films were successfully targeted for covalent binding of gold nanoparticles, biotin and enzyme (β-galactosidase) using maleimide chemistry. Modification of already assembled films was suggested a better option for coupling of lager molecules that otherwise could interfere with the silk assembly process.

Modification of spider silk films has also been achieved by covalent binding of glycopolymers for generation of a biomimetic silk surface (Hardy *et al.*, 2014). In the two-step approach for glycopolymer binding, an azidopropylamine linker was first bound to exposed carboxylic groups on the silk film surface using EDC/NHS coupling. Next, glycopolymers were covalently coupled to the azidopropylamine linker via cyclo-addition chemistry. The covalently bound glycopolymers exhibited binding affinity for proteins derived from the extracellular matrix (ECM) with importance for cell adhesion and therefore silk/glycopolymer films displayed increased cell adhesion properties compared to unmodified silk films.

Covalent attachment of oligonucleotides to recombinant spidroins was recently presented as a concept for functionalization of spider silk (Humenik & Scheibel, 2014). Self-assembled fibrils produced from silk/DNA-biotin conjugates were able to bind neutravidin-modified gold nanoparticles through oligonucleotide complementarity.

1.4.5 Covalent: Genetic fusion

Low prevalence of reactive amino acid residues in spider silk proteins and silk fibroin (Murphy & Kaplan, 2009; Vollrath, 1999) may make functionalization by chemical modification less efficient. Moreover, chemical modification of amino acid residues can be considered as a random event with low controllability as the exact position of the coupled residues most often cannot be pre-determined, at least if more than one reactive residue is present in the protein sequence. As an alternative, covalent modification by genetic engineering has been used to add new functions to recombinant spider silk

(Widhe *et al.*, 2013; Wohlrab *et al.*, 2012; Bini *et al.*, 2006) and recombinant silk fibroin (Hino *et al.*, 2006; Inoue *et al.*, 2005). In the genetic fusion approach for silk functionalization the DNA sequence encoding the silk protein is fused to the DNA sequence encoding the functionalization domain (*e.g.*, short peptide or protein) using recombinant DNA technology. The resulting fused DNA sequence is inserted into an appropriate production host system and expressed as a silk fusion protein, in which the silk protein part and the functionalization protein part are covalently linked by a stable peptide bond. Furthermore, recombinant production of silk fusion proteins allow for every produced silk molecule to possess a covalently linked functionalization molecule.

Spider silk proteins have been genetically fused to short cell-binding motifs (*e.g.*, RGD and IKVAV) for subsequent expression in *E. coli*. A CRGD peptide, containing the integrin recognition sequence RGD, was fused to a gene encoding a recombinant spidroin derived from MaSp1 of *N. clavipes* (Bini *et al.*, 2006). After expression and purification of the spider silk fusion protein (49 kDa), soluble CRGD-silk proteins were processed into films by casting from a HFIP solution, followed by methanol treatment. Structural analysis of casted films revealed an increase in β-sheet structure upon treatment with methanol, corresponding to what was observed for films prepared from the non-functionalized spidroin. In addition, fibers from CRGD-silk were prepared by electrospinning from a HFIP solution, and conversion to β-sheet structure was also observed for methanol treated fibers. However, analysis of functionalized CRGD-silk films for cell response revealed as good outcome for the CRGD-silk variant as for non-functionalized silk. In another study, the partial spider silk protein 4RepCT was used for functionalization with the cell-binding motifs RGD, IKVAV and YIGSR, respectively (Widhe *et al.*, 2013). Purified silk fusion proteins were processed into water-stable fiber, film and foam from a physiological-like buffer (pH 8). By FTIR analysis the same β-sheet rich structure was observed for the functionalized silk materials as for the corresponding non-functionalized materials. Furthermore, the exposure of the covalently linked peptides in all material formats was evident from cell adhesion analysis using different human primary cells types. In another study, the spidroin eADF(C16) was genetically linked to different variants of short peptides, all including the RGD motif, and used to produce functionalized silk films (Wohlrab *et al.*, 2012). The films showed no influence from peptide fusion on the secondary structure, however, the impact on cell attachment and proliferation was improved due to the RGD modification.

In a series of studies the design and production of silk fusion proteins applicable in gene delivery systems have been demonstrated (Numata *et al.*,

2011; Numata & Kaplan, 2010b; Numata *et al.*, 2009). A spider silk repeat unit derived from MaSp1 of *N. clavipes* was genetically engineered to contain various numbers of consecutive lysine residues (poly-lysine). Expressed and IMAC purified silk fusion proteins (silk-lys) was allowed to bind plasmid DNA (pDNA) in solution, an interaction mediated by attractive electrostatic interactions between positively charged lysines and negatively charged pDNA. The formed silk-lys/pDNA complexes were then used for cell transfection, delivering the genetic information contained in the pDNA. Moreover, additional functionalization of the silk-lys protein was achieved by genetic fusion of the silk-lys sequence to various cell membrane destabilizing peptides (CMDP), resulting in silk-lys-CMDP proteins showing increased transfection efficiency. Finally, the CMPD peptides were replaced by tumor homing peptides (THP) generating silk-lys-THP for tumor specific gene delivery.

Spider silk bioconjugates to induce mineralization has been attempted. A chimeric spider silk protein (silk-R5) composed of repeated sequences of one repeat unit from MaSp1 of *N. clavipes* fused to a gene (denoted R5) derived from the silaffin protein was constructed (Foo *et al.*, 2006). The R5 peptide is previously known for its ability to precipitate silica in silicification reactions. Casted films and electrospun fibers were produced from lyophilized silk-R5 protein dissolved in HFIP and subsequently treated with methanol. The effect from added R5 peptide was obvious as silica particles were formed onto the silk-R5 materials in mineralization experiments. Using the same R5 peptide (Mieszawska *et al.*, 2010) or other peptides with mineralization abilities (Belton *et al.*, 2012; Gomes *et al.*, 2011a; Huang *et al.*, 2007) various aspects of mineralization processes using recombinant spider silk fusion proteins have been investigated.

Introduction of antimicrobial properties in genetically engineered spider silk materials has also been reported. One example is genetic fusion of the antimicrobial peptides (AMP) hepcidin, human neutrophil defensin 2 and 4 to a spidroin sequence derived from MaSp1 of *N. clavipes* (Gomes *et al.*, 2011b). Silk-AMP composite proteins (~25 kDa) were expressed in *E. coli*, purified using IMAC and lyophilized. Films of all three silk-AMP proteins were prepared by casting from solutions of silk-AMP in MilliQ water, followed by drying and methanol treatment. Analysis of films using FTIR revealed an increase in β-sheet content to approximately 40% in all silk fusion films, comparable to non-functionalized films. Activity tests using a radial diffusion assay revealed bactericidal activity in the soluble silk-AMP proteins, however this was not shown for the formed silk films.

Peptide-based recognitions motifs have also been genetically linked to spider silk proteins in order to induce silver and uranium binding properties

(Krishnaji & Kaplan, 2013; Currie *et al.*, 2011). Silver-binding silk chimeric proteins were achieved by fusion of spider silk repeat units derived from MaSp1 of *N. clavipes* to two silver-binding peptides, Ag-4 and Ag-P35, respectively (Currie *et al.*, 2011). Soluble silk-Ag-4 proteins were assessed for silver binding in an $AgNo_3$ solution and observed precipitation of silver nanoparticles confirmed the ability of the silk fusion protein to bind silver. Moreover, casted silk-Ag films could form silver nanostructures on their surfaces when subjected to a silver solution, as a result of silver binding. In addition, these silver-coated silk surfaces showed antimicrobial properties by inhibiting the growth of gram-negative and gram-positive and thus, coating of materials with silk-Ag proteins were suggested as a way of achieve biomedical antimicrobial coatings. As a final example of using gene technology to achieve spider silk fusion proteins, a silk protein with uranium binding capability can be mentioned (Krishnaji & Kaplan, 2013). A uranyl-binding peptide was fused to a recombinant spidroin (based on MaSp1, *N. clavipes*) and subsequently expressed as a silk fusion protein. Binding of uranium ions was confirmed for the soluble silk fusion protein, however, any attempts to create solid silk materials were never made. Remediation of areas polluted with uranium was an envisioned application for the produced silk-uranium binding protein.

Use of genetic engineering approaches have also been utilized to design and express silk fibroin chimeric proteins. Human basic fibroblast growth factor (bFGF) has been fused to the silk fibroin L-chain gene, followed by expression of the fibroin-bFGF fusion protein in transgenic *B. mori* (Hino *et al.*, 2006). A silk solution composed of natural fibroin and fibroin-bFGF was successfully extracted from cocoons spun by the transgenic silkworms. Interestingly, polystyrene wells coated with fibroin-bFGF supported growth of human umbilical vein endothelial cells. Another example of using transgenic silk worm is the production of fibroin L-chain fused to green fluorescent protein (GFP) (Inoue *et al.*, 2005). The fibroin-GFP fusion protein was expressed in high yield, and it was determined that around 10% of the total silk fibroin content in a single cocoon consisted of the fibroin-GFP fusion protein.

1.5 Applications of functionalized silk

The techniques discussed in the former section to add new functional properties to silk have opened up for new possible applications of functionalized silk-based materials. Areas in which the combination of silk with additional (bio)activities have shown potential are so far mostly related to medicine and biotechnology (Borkner *et al.*, 2014; Yucel *et al.*, 2014; Tao *et al.*, 2012b; Leal-Egana & Scheibel, 2010; Numata & Kaplan, 2010a; Wang *et*

al., 2006). In this section, the use of functional silk materials in some selected application fields will be presented. Most effort has been made towards applications in the areas of cell scaffolds and drug delivery, although more technical applications as biosensors and electronics have also been explored, and will be discussed herein since it is more in line with the focus of the work presented in this thesis.

1.5.1 Cell scaffolds

In the human body cell-binding motifs present in the ECM proteins (*e.g.*, laminin, fibronectin, collagen, vitronectin) are recognized by cell-surface integrins, thereby mediating an interaction between cells and ECM (Simon-Assmann *et al.*, 2011; Chillakuri *et al.*, 2010; Heino, 2007; Schvartz *et al.*, 1999; Johansson *et al.*, 1997). In trying to mimic the environment provided by the ECM, bioactive molecules like cell-binding peptides and growth factors have been incorporated into silk-like materials to create functional cell scaffolds with cell interacting properties (Dinis *et al.*, 2014; Gil *et al.*, 2013; Widhe *et al.*, 2013; Wohlrab *et al.*, 2012; Bini *et al.*, 2006; Sofia *et al.*, 2001). Recombinant spider silk scaffolds functionalized with cell-binding motifs, for example RGD (Widhe *et al.*, 2013; Wohlrab *et al.*, 2012), were mentioned earlier, and the incorporated motifs showed positive effects on cell characteristic features as adherence, proliferation and viability. The established processing techniques for production of silk materials offer the possibility to achieve scaffolds with both two-dimensional and tree-dimensional geometry, of which the latter best resembles the environment experienced by a cell in the human body. Moreover, to achieve realistic ECM-like conditions the use of multifunctional scaffolds, combining different cell-binding motifs and/or growth factors, hold future promise.

An example of silk in tissue engineering is the use of optically transparent silk films for corneal tissue formation (Lawrence *et al.*, 2009; Lawrence *et al.*, 2008). Thin free-standing films of silk fibroin showing optical transparency were fabricated for intended use in cornea tissue engineering (Lawrence *et al.*, 2009). Fabricated silk films promoted adherence, proliferation and corneal extracellular matrix expression of human and rabbit corneal fibroblasts. Therefore, it was concluded that the scaffold holds promise in promoting the formation of corneal tissue. Moreover, another study showed successful incorporation of bioactive molecules (hemoglobin and peroxidase) into the same silk films, resulting in optically active silk films (Lawrence *et al.*, 2008).

More about the use of recombinant spider silk and silk fibroin scaffolds for interaction with cells, for example in tissue engineering, has been reviewed

elsewhere (Schacht & Scheibel, 2014; Wu *et al.*, 2014; Kasoju & Bora, 2012; Widhe *et al.*, 2012; Zhang *et al.*, 2009; MacIntosh *et al.*, 2008).

1.5.2 Drug delivery

There are a few important aspects to consider when designing a carrier material for drug delivery. The processing of the carrier material, together with the physicochemical, biological and pharmacological properties are of major importance, and in relation to this, silk can offer beneficial properties (Yucel *et al.*, 2014). Advantageous features of silk, such as processing flexibility, biodegradability and adjustment of drug release profile, make silk a potent carrier vehicle for drug delivery (Numata & Kaplan, 2010a). Silk fibroin has therefore been investigated as a carrier material for sustained release of various small molecule drugs and biological drugs after processing of silk into films (Pritchard *et al.*, 2013; Uebersax *et al.*, 2007), porous scaffolds (Wilz *et al.*, 2008; Karageorgiou *et al.*, 2006), microcapsules (Cheema *et al.*, 2007), microspheres (Wang *et al.*, 2009; Wenk *et al.*, 2008) and hydrogels (Elia *et al.*, 2013; Guziewicz *et al.*, 2011). Further aspects on the use of silk fibroin as carrier for drug delivery is discussed more thoroughly elsewhere (Wenk *et al.*, 2011).

Also silk materials processed from recombinant spider silk proteins have been proven suitable as vehicles for drug delivery. This has been proven by release of small model drug molecules (Hardy *et al.*, 2013; Blum & Scheibel, 2012; Lammel *et al.*, 2011) and biological model drugs (Hofer *et al.*, 2012). Knowledge acquired from such investigations regarding parameters for drug loading and release profiles can act as a foundation for design of more highly tunable spider silk drug carriers.

1.5.3 Biosensors

Attempts have been made to create biosensors by utilizing silk materials with associated sensing elements. As an example, biosensors using silk fibroin with enzymes as the sensing element have been demonstrated. It should be mentioned that silk has been reported to mediate an overall stabilizing effect on enzymes (Pritchard *et al.*, 2012; Lu *et al.*, 2009), why the combination of silk and enzymes into a biosensor system seem like a promising strategy. Silk-based biosensors for sensing of glucose have been achieved by functionalizing silk fibroin with glucose oxidase (Zhang *et al.*, 1998b; Demura *et al.*, 1989). In both cases silk fibroin membranes with non-covalently attached glucose oxidase enzymes could detect glucose substrate using electrical read-out. Similarly, horseradish peroxidase was combined with silk fibroin membranes in order to generate a biosensor for detection of hydrogen peroxide (Qian *et al.*,

1996). Rapid and sensitive detection of hydrogen peroxide was achieved as the sensor system reached a steady-state signal within 40 second, and with a limit of detection at 0.1 μM. Furthermore, the introduction of "New methylene blue N" reagent in the biosensor system effectively helped out in transferring electrons generated by the enzyme reaction to the electrode. A biosensor system for detection of uric acid has also been presented and was composed of a silk fibroin membrane with immobilized uricase (Zhang *et al.*, 1998a). The biosensor responded to uric acid solutions with linearity in the response for concentrations of 0.2-1.0 mM of uric acid. Interestingly, measurements of urate levels in human serum and urine were also feasible.

A subgroup of biosensors is the immunosensors, taking advantage of the specificity in the biological interaction between antigen and antibody. As a demonstration on the use of immunosensor technology in combination with silk-based materials the antigenic peptide NS5A-1, derived from hepatitis C virus, was combined in alternating layers with silk fibroin onto electrodes (Moraes *et al.*, 2013). Upon addition of anti-NS5A-1 antibody signal was achieved using electronic read-out, demonstrating the applicability of silk-based immunosensors for specific detection. When immobilizing an active biomolecule to a surface it is of outermost importance that the immobilization *per se* is not altering the biomolecule conformation and, in turn, not destroys the basis for antigen-antibody interaction (Teles & Fonseca, 2008). In a previous study it was shown that a primary antibody immobilized to silk fibroin films was detected to a larger extent by a secondary antibody when non-covalently entrapped in the films compared to covalent coupling (Lu *et al.*, 2011). The suggested explanation for this observation was, in part, due to the retained conformation of the primary antibody from the mild reaction conditions, as compared to covalent coupling. This finding could be considered in design of future silk-based immunosensors.

The lack of reported sensors based on recombinant spider silk may well be changed in the near future due to the advancements in creating functionalized spider silk. Particularly, the design of bioactive spider silk from genetic fusion can pave the way for high sensitivity biosensors, as a result from being able to functionalized every spider silk molecule.

1.5.4 Electronics

Processing of silk fibroin materials for intended use in electronics has been proposed (Tao *et al.*, 2012b; Kim *et al.*, 2010) and two examples will be given below. To add electronics onto a silk template may not fit very well into the term "functionalization" of silk as it has been used so far, although I think these examples may broaden the perspective on the applicability of silk

materials. Moreover, the combination of silk and electronics could be turned into advanced implantable sensor devices, particularly if further functionalized with active biomolecules.

Standard semiconductor fabrication technology has been used to print electronic circuits in silicon (Kim *et al.*, 2009). The printed electronics pattern was then transferred onto the surface of free-standing silk fibroin films in a series of steps, generating a silk-electronics device. An advantage of transferring the silicon electronics onto the silk material is the generation of a flexible structure, a great advantage for the purpose of implantable devices. Another benefit from such system is that the silk carrier is resolvable and can eventually be resorbed within the body. The concept of implantable electronics was further evaluated in animal studies.

Another interesting application is the construction of silk-electronics sensors for food monitoring (Tao *et al.*, 2012a). Silicon structures that can respond electromagnetically to physical changes (*e.g.*, changes in the dielectric constant) in the nearby environment can be fabricated using standard semiconductor fabrication methods. Such structures were transferred to silk fibroin films, whereupon the entire flexible device was glued onto fruits. Physical and chemical changes during fruit ripening were monitored using the silk-electronics device, and applicability in monitoring of food quality was envisioned.

2 Present investigations

2.1 Aims of the present studies

The overall aim of the present studies has been to explore the possibility to functionalize the recombinant spider silk protein 4RepCT, and thereby equipping materials made thereof with new properties. Specific attention was paid to a functionalization technique in which a functional protein domain is fused to 4RepCT at the DNA level. This thesis more specifically aims at:

- Investigating the staining of 4RepCT fibers with two conjugated polyelectrolytes for realizing fibers with electrical and fluorescence properties (**Paper I**).

- Exploring genetic linkage as a technique for functionalization of 4RepCT with affinity protein domains and evaluating the functionality of formed silk materials (**Paper II**).

- Investigating the possibility of using the yeast *Pichia pastoris* as a host for expression and secretion of the functionalized silk fusion protein Z-4RepCT (**Paper III**).

- Evaluating the concept of enzyme-silk by studying the catalytic ability of functionalized silk materials in which an enzyme has been genetically linked to the spider silk modules 4RepCT and CT (**Paper IV**).

2.2 Results and Discussion

2.2.1 Paper I: Fibers of recombinant spider silk stained with conjugated polyelectrolytes show electrical conductivity and fluorescence properties.

The easiest way to functionalize a silk fiber is by utilizing non-covalent interactions between the fiber and the molecule to be associated, such as in plain adsorption (coating). Using appropriate coating conditions, fibers could simply be immersed into a solution containing the molecules to be coated onto the fiber. Functionalization of silk fibers by coating has previously been demonstrated feasible for production of, for example, electrically conducting silk fibers (Xia & Lu, 2008; Kim *et al.*, 2007). In these cases the silk fibers were originated from *B. mori* (Xia & Lu, 2008; Kim *et al.*, 2007) and from natural spider dragline silk (Kim *et al.*, 2007). Moreover, the coating technique has in a similar way been used with inorganic compounds to induce fluorescence properties of natural spider dragline silk fibers (Chu & Sun, 2007). In this paper (paper I) the aim was to explore the possibility to use recombinant 4RepCT spider silk fibers as template for coating with conjugated polymers in an attempt to create fibers with electrical and fluorescence properties.

For induction of electrical conductivity, coating with the conjugated polyelectrolyte PEDOT-S, poly(4-(2,3-dihydrothieno[3,4-*b*]-[1,4]dioxin-2-yl-methoxy)-1-butanesulfonic acid, sodium salt), was performed. In general, a polyelectrolyte exhibits both polymer and electrolyte properties. Features of PEDOT-S turning it into a suitable candidate for coating in this study are its good electrical conductivity in the solid state in combination with its ability of being fully water-soluble (Karlsson *et al.*, 2009). Moreover, PEDOT-S has previously shown capable of binding to β-rich protein structures (Hamedi *et al.*, 2008).

To survey conditions affecting the PEDOT-S staining of 4RepCT fibers, three staining parameters were investigated. There was a difference in the rate of fiber staining when comparing high (10 g L^{-1}) and low (0.1 g L^{-1}) concentrations of PEDOT-S, where the interaction of the two materials were visualized by the change in fiber color from white (unstained) to deep blue (stained). In other words, the same degree of staining could be achieved using a dilute PEDOT-S solution as for a more concentrated one, but the staining rate was reduced for dilute solutions. The second parameter to influence the rate of staining was the temperature. It turned out that when elevating the temperature to 90°C, staining could be accomplished in 1-2 h compared to two weeks at room temperature. Thus, so far, it was concluded that efficient PEDOT-S

staining of 4RepCT fibers could be achieved in 10 g L^{-1} PEDOT-S for one hour at 90°C.

The final staining parameter to be evaluated was the pH of the staining solution. When varying the pH of the staining solution between 2 and 11, an increase in fiber staining was observed with a decrease in pH, with most efficient staining achieved at pH 2. This observation hints that favorable electrostatic interactions may play a crucial role in the interaction between the two components. The isoelectric point (pI) of 4RepCT has a calculated value of 8.9 (Hedhammar *et al.*, 2008), meaning that for pH > 8.9 the fibers are negatively changed and for pH < 8.9 they are positively charged. The increasingly positive charge for fibers at pH < pI could result in favorable electrostatic interactions with the negatively charged PEDOT-S. Also, fibers stained at pH 2 lose PEDOT-S staining, seen by a major loss of color, when exposed to a solution of pH 13, indicating repulsive electrostatic interactions from both materials being negatively charged. It should, however, be emphasized that fibers could be slightly stained even at pH 11, pointing towards the fact that electrostatic interactions may not be acting alone in the staining process, something that has been previously suggested in the interaction between polypeptide structures and conjugated electrolytes (Herland *et al.*, 2008; Nilsson *et al.*, 2006; Nesterov *et al.*, 2005).

Next, the effect on fiber structure and mechanical properties from staining was explored. Microstructure of stained and air-dried fibers was investigated using X-ray diffraction to determine whether the staining process in itself had altered the fiber structure. Compared to unstained fibers, the stained ones showed the same diffraction pattern, concluding that the overall fiber structure was unaffected by the staining. Mechanical strength of stained, air-dried fibers was examined using differential mechanical analysis (DMA) and indicated a slightly decreased fracture stress for stained fibers. Interestingly, optical microscopy of a cross-section of a stained fiber showed staining throughout the fiber, and not only on the surface. This could explain the decreased mechanical properties, in that PEDOT-S situated between fiber bundles would to some extent hinder β-sheet hydrogen bonding between those upon air-drying. On the other hand, bulk fiber staining could give rise to increased electrical properties, compared to only surface dyeing. Electrical conductivity was measured to $(8 \pm 2) \cdot 10^{-4}$ S cm^{-1} for fibers stained at 90°C, $(7 \pm 2) \cdot 10^{-5}$ S cm^{-1} for staining at room temperature and $< 10^{-7}$ S cm^{-1} for unstained fibers. The highest conductivity values measured for stained 4RepCT fibers was approximately one order of magnitude lower than what has been previously reported for natural spider silk fibers coated with carbon nanotubes and silkworm silk fibers coated with PEDOT (Xia & Lu, 2008; Kim *et al.*, 2007).

Interestingly, conducting polymer templates have been used to electrically stimulate cells for enhanced neurite outgrowth (Schmidt *et al.*, 1997), increased proliferation of smooth muscle cells (Rowlands & Cooper-White, 2008) and to tune the shape and function of endothelial cells (Wong *et al.*, 1994). In one of the studies, an electrically conductive material (~$2.3 \cdot 10^{-3}$ S cm^{-1}) composed of polypyrrole and hyaluronic acid was used to support enhanced growth of smooth muscle cells stimulated with a 50 µA electrical current (Rowlands & Cooper-White, 2008). The fairly low magnitude of the electrical current used for stimulation holds promise for the conductive 4RepCT fibers presented herein as conducting polymers for cell stimulation.

The conjugated polyelectrolyte PTTA, poly(thiophene acetic acid), was used for coating of 4RepCT fibers, generating fluorescent fibers. In a similar way to what described above, it was concluded that staining time and pH were affecting the staining efficiency. Most efficient staining was achieved after two hours at 90°C. Effect of solution pH on staining was also evaluated and in the range of pH 7-11 that was analyzed, highest levels of fibers fluorescence was gained from the interval of pH 7-9. In contrast, fluorescence was principally absent for pH 11, whereas weak fluorescence was identified at pH 10. For those reasons it was suggested that electrostatic interactions play the main role in the interaction between the two materials, as 4RepCT is positively charged for pH < 8.9, while PTAA is negatively charged. Moreover, weak fluorescence at pH 10 again implies the impact of other interactions, besides the electrostatic ones.

Summary (Paper I):

- Staining of recombinant spider silk (4RepCT) fibers with the conjugated polyelectrolyte PEDOT-S induced bulk-electrical fiber properties.
- Polyelectrolyte concentration and pH of staining solution affected the staining efficiency. The staining temperature also had an influence.
- The fiber-polyelectrolyte interaction appeared to be mainly mediated through electrostatic interactions.
- Microstructure of stained fibers was not affected by staining, but the mechanical properties were slightly impaired.
- The generality of the 4RepCT staining method was shown by fiber staining with the conjugated polyelectrolyte PTAA, generating fluorescent fibers.

2.2.2 Paper II: Silk fusion proteins with fold-dependent affinity domains genetically linked to recombinant spider silk have the ability to self-assemble into fibers with affinity properties.

The work in paper I revealed functionalization of recombinant spider silk fibers by physical staining with polyelectrolytes mediated by non-covalent interactions. If functionalization with peptides or protein molecules instead is desirable, attachment via covalent coupling may be a more attractive approach in order for the attached molecules to be kept in place over time. Covalent coupling of cell-binding peptides and larger protein entities (*e.g.,* growth factors and affinity domains) to silkworm fibroin has previously been achieved using reactive amino acids for coupling (Wang & Kaplan, 2011; Karageorgiou *et al.*, 2004; Chen *et al.*, 2003; Sofia *et al.*, 2001). Concerning spider silk, recombinant spider silk proteins have, interestingly, been engineered at the genetic level to contain additional sequence segments adding various functionalities. The expressed spider silk fusion proteins reported have attained additional features due to genetic linkage of sequences encoding, for example, cell-binding peptides (Widhe *et al.*, 2013; Wohlrab *et al.*, 2012; Bini *et al.*, 2006), mineralization inducing sequences (Gomes *et al.*, 2011a; Mieszawska *et al.*, 2010; Huang *et al.*, 2007) and antimicrobial peptides (Gomes *et al.*, 2011b). In line with this, the aim of this paper (paper II) was to investigate the possibility of using genetic linkage as a technique for functionalizing the recombinant spider silk protein 4RepCT with protein domains.

As domains for functionalization of 4RepCT, four protein domains with fold-dependent affinity properties were selected. The two domains Z and C2 both bind immunoglobulin (Ig) G, but have different fold. Domain Z is composed of 58 amino acid residues folded into a three-helix bundle (Nilsson *et al.*, 1987), whereas the C2 domain consists of 56 residues attained in a mixed β-sheet/α-helix fold (Sauer-Eriksson *et al.*, 1995). The third affinity domain for functionalization was the albumin binding domain (ABD), folded as a three-helix bundle composed of 46 amino acid residues (Kraulis *et al.*, 1996). Thus, the Z and ABD domains are folded similarly, but with different binding selectivity towards IgG and serum albumin, respectively. The last domain included in this study was the 159-residue streptavidin-derived M4 domain folded into a β-sheet barrel, and showing affinity towards biotin (Wu & Wong, 2005).

Genes for all four silk fusion proteins (Z-4RepCT, C2-4RepCT, ABD-4RepCT and M4-4RepCT) were engineered by linkage of DNA encoding the respective affinity domain to the 4RepCT gene. The silk fusion proteins were then successfully expressed in *E. coli* and recovered as soluble proteins after IMAC purification. Interestingly, macroscopic fibers were self-assembled from

soluble protein for all silk fusion variants, implying that the addition of affinity domains does not affect the silk assembly properties of 4RepCT. It should be noted that the molecular sizes of linked domains ranges from 5-7 kDa (Z, C2 and ABD) to 17 kDa (M4), compared to the size of 23 kDa for 4RepCT. One observation implying that the fiber formation process was not affected for the silk fusion proteins was the elapsed time until macroscopic fibers were visible by eye, which was roughly the same as for non-functionalized 4RepCT. Moreover, light microscopy revealed similar macroscopic appearances for fibers made from silk fusion proteins compared to 4RepCT fibers. Finally, analyses of secondary structure content by Fourier transform infrared spectroscopy (FTIR) showed that both fibers and films made from silk fusion protein possessed the same β-sheet signal typical for 4RepCT in silk form.

To investigate the affinity properties of produced silk fusion materials, different routes were taken. For analysis of functionality and selectivity of IgG-binding to Z-4RepCT and C2-4RepCT silk, a complex protein solution constituted by rabbit serum was used to immerse the silk materials, followed by wash, low-pH release of IgG and analysis by non-reducing SDS-PAGE. Release fractions revealed a protein band around 140 kDa, corresponding to intact IgG, for both fibers and films of Z-4RepCT and C2-4RepCT. A similar analysis using the complex protein solution of human plasma revealed a single protein band positioned in-between 50-70 kDa, corresponding to albumin, for films and fibers of ABD-4RepCT. The binding of biotin to M4-4RepCT films was instead studied by using biotinylated DNA in a complex DNA mixture, followed by wash and enzymatic release of captured biotinylated DNA. Released fraction analyzed with agarose gel electrophoresis revealed a single band corresponding to the size of biotinylated DNA. These results indicate the maintained biological activity of the added protein domains to bind their intended target molecules although produced in fusion with, and thus covalently linked to, the spider silk module 4RepCT. It is also shown that the fold-dependent binding of target molecules to silk fusion materials is selective. Moreover, it could be demonstrated that the binding of target molecules to silk fusion films was distributed over the whole film surface area by analyzing the binding of directly or indirectly fluorophore-labeled targets.

In attempt to create silk materials with dual functionality, fibers and films made from a mixture of soluble Z-4RepCT and ABD-4RepCT were prepared. Human plasma was used as source for both IgG and albumin to incubate with the mixed Z-4RepCT/ABD-4RepCT silk materials. Release fractions from fibers and films showed two distinct protein bands, corresponding to IgG and albumin, respectively. Thus, it is proven possible to create silk materials from differentially functionalized 4RepCT with multiple functionalities. As the

genetic approach for functionalization of 4RepCT used in this paper allows for every expressed 4RepCT molecule to have an attached functional domain, the opportunity to design silk materials with predefined ratios of functional domains is envisioned.

The applicability of functionalized 4RepCT materials was shown for presentation of growth factors on Z-4RepCT films. The films were first decorated with antibodies targeting human vascular endothelial growth factor (VEGF), using the affinity of the Z domain to bind IgG. Next, the antibodies were used to capture fluorophore-labeled human VEGF, followed by visualization using fluorescence microscopy. The entire surface area of the films displayed fluorescence from bound VEGF, as opposed to non-fluorescent control films of either made from non-functionalized 4RepCT or from Z-4RepCT but with the capture antibody excluded. This approach to expose bound growth factors to the surroundings could be beneficial in advanced cell culturing, especially in an economical context, as they will remain bound upon media exchange and not needed to be added again. However, this approach could be generalized for presentation of a very wide range of biological molecules, the only limitation being the availability of an antibody directed against the molecule to be presented. Furthermore, the use of M4-4RepCT films for presentation of active enzymes was shown by the capture of biotinylated xylanases that after binding to the films displayed maintained enzymatic activity. Likewise, the generality for presentation of biotinylated molecules by M4-4RepCT materials extends beyond enzymes and is applicable to any biomolecule possible to biotinylate.

Summary (Paper II):

- A strategy for functionalization of 4RepCT spider silk with functional protein domains via genetic linkage is presented.
- Silk-fusion proteins designed by genetic linkage of functional protein domains (Z, C2, ABD, M4) to 4RepCT could self-assemble into silk-like fibers.
- Silk materials showed selective and fold-dependent affinity towards their respective target molecule.
- Combined IgG and albumin affinities were achieved in silk materials prepared from a mixture of Z-4RepCT and ABD-4RepCT.
- Silk films decorated with antibody-bound growth factors were realized using the antibody-binding property of Z-4RepCT.

2.2.3 Paper III: The affinity silk fusion protein Z-4RepCT can be expressed and secreted by the methylotrophic yeast *Pichia pastoris*, and obtained as a mixture of full-length and degraded proteins upon purification.

In paper II the production of genetically functionalized spider silk materials was demonstrated. Silk fusion proteins expressed in *E. coli* showed maintained ability of the 4RepCT module to self-assemble into silk fibers. In addition, the covalently linked affinity domains showed affinity for their intended target molecules. For future applications of functionalized 4RepCT-based materials an increased capacity in the production of these materials would be desirable. One way of achieving this goal could be to use the methylotrophic yeast *P. pastoris* as expression host, allowing for production and secretion of large quantities of recombinant proteins. Earlier attempts to express and secrete silk-like and silk-collagen-like proteins using *P. pastoris* have showed upon g L^{-1} levels of secreted protein (Golinska *et al.*, 2014; Wlodarczyk-Biegun *et al.*, 2014; Werten *et al.*, 2008). With this in mind, the aim of the current paper (paper III) was to explore the use of *P. pastoris* as host for heterologous expression and secretion of the silk fusion protein Z-4RepCT.

As choice of expression vector, a pPICZαA vector was used to incorporate the gene encoding Z-4RepCT prior to transformation into methylotrophic *P. pastoris* (Cregg *et al.*, 1985). The *AOX1* promoter region (Tschopp *et al.*, 1987) provided in the vector allows for methanol-induced expression. Also present in the vector is the α-factor signal sequence, derived from *Saccharomyces cerevisiae*, allowing for secretion of expressed proteins into the growth medium (Waters *et al.*, 1988; Brake *et al.*, 1984; Julius *et al.*, 1984).

To start the investigation of Z-4RepCT production in *P. pastoris*, three different expression parameters were examined. The first parameter, expression temperature, was set at 20°C and 25°C at constant pH 6. As shown from analysis of secreted proteins by Western blot against hexa-histidine, strongest intensity for candidate Z-4RepCT bands positioned at 35-50 kDa was achieved at a temperature of 20°C. These bands got even stronger in intensity when increasing the pH value for expression to pH 7. Thus it was concluded that both temperature and pH influence the expression and secretion of Z-4RepCT. Moreover, it was obvious that the expression time also had an impact on the amount of secreted silk fusion protein. When protein secretion was analyzed during 96 h of expression, Western blot analysis revealed an increase in band intensities up to 24 h, and then the band intensities gradually decreased. It was suggested that protein degradation by *P. pastoris* proteases and/or aggregation of Z-4RepCT caused this gradual decrease. Furthermore, the protein identity of secreted Z-4RepCT protein was confirmed by mass

spectrometry analysis, as peptides from both the Z domain and from 4RepCT was identified.

As degradation of Z-4RepCT by proteases present in the *P. pastoris* growth medium was suggested as a possible problem, the effect of *P. pastoris* conditioned growth medium on non-functionalized 4RepCT protein (expressed in *E. coli*) was investigated. Purified 4RepCT was added to *P. pastoris* conditioned growth medium and the occurrence of degradation bands over time was studied by SDS-PAGE. After one hour of incubation a protein band at around 20 kDa (calculated molecular weight of 4RepCT is 23 kDa) appeared that was indicative of protease-mediated degradation. The same band appeared when the metalloprotease inhibitor EDTA was added from the beginning. However, this protein band did not appear when the aspartic protease inhibitor Pepstatin A instead was present, indicating the possibility to use Pepstatin A to limit 4RepCT degradation by proteases present in the *P. pastoris* growth medium.

The investigation moved on by trying to purify *P. pastoris* secreted Z-4RepCT protein by IMAC. SDS-PAGE analysis of bound and eluted proteins after IMAC purification revealed a number of different bands of various sizes. However, there were three bands showing considerable staining intensity. One band was positioned at 40 kDa and thus, a candidate of representing Z-4RepCT whose calculated molecular weight is 32 kDa. The other two bands were situated at 10 and 15 kDa, respectively. Corresponding Western blot analysis again revealed the same bands in-between 35-50 kDa as previously seen, although the 10 and 15 kDa bands pronounced in SDS-PAGE were not visible at all in Western blot. Mass spectrometry was used to elucidate the identity of the three bands of strong intensity from SDS-PAGE. It turned out that the candidate band for full-length Z-4RepCT at 40 kDa contained peptides from both the Z domain as well as from 4RepCT, implying successful purification of secreted silk fusion protein. However, also in both of the low molecular-weight bands (10 and 15 kDa), peptides from the C-terminal part of 4RepCT were identified. Moreover, mass spectrometry of soluble protein after IMAC purification revealed a 77-residue polypeptide fragment corresponding to the very C-terminal region of Z-4RepCT that was partly truncated in the His_6 tag. Analysis of the same type of sample after trypsin treatment revealed another fragment, and together the two identified fragments suggest a specific site in the CT domain of 4RepCT (QVM/AALP) which was subjected to cleavage. From these results the conclusion was made that a mixture of full-length and degraded Z-4RepCT can be purified from *P. pastoris* growth medium.

The previously observed effect from the protease inhibitor Pepstatin A on degradation of 4RepCT added to *P. pastoris* growth medium was explored in

an attempt to limit the degradation products from purified Z-4RepCT. Ratios between band intensities from full-length protein (40 kDa) and from the two low molecular-weight bands (10 and 15 kDa) after IMAC purification was investigated after expression without protease inhibitor and with added Pepstatin A and Complete Protease Inhibitor Cocktail, respectively. As estimated intensity ratios varied a lot for the different occasions of expression with added protease inhibitors, the effect of the protease inhibitors on Z-4RepCT degradation was inconclusive.

As glycosylation of expressed proteins in *P. pastoris* is a well-known phenomenon, the presence of N-linked and O-linked glycosylations in purified Z-4RepCT protein was examined. From SDS-PAGE of soluble protein treated with the deglycosylation enzyme PNGase F, removing N-linked glycosylation, a pronounced band shift in the 10 kDa and 15 kDa degradation bands was observed. After PNGase F treatment, the intensity of the 15 kDa band was decreased, whereas the intensity for the 10 kDa band instead had increased. The removal of N-linked glycans upon PNGase F treatment was also evident from mass spectrometry analysis, which was shown by a decrease in identified glycopeptides upon this treatment. However, some degree of glycosylation still remained after treatment with PNGase F, suggesting the presence of also O-linked glycans. A closer investigation of the tryptic fragment LSSPSAVSR by mass spectrometry suggested the presence of O-linked glycans in a pattern of LSSPSAVSR + (1-3) hexose. Thus, it is demonstrated that expressed and secreted Z-4RepCT protein contains both N-linked and O-linked glycosylation.

The observed alterations in the CT domain of 4RepCT, subjected to both cleavage and glycosylation, could contribute to the fact that no self-assembly into silk-like fibers was observed for purified Z-4RepCT. As a specific position in the CT domain subjected to cleavage was identified, the use of point mutations within this site could possibly prevent the cleavage and thereby reveal the impact of cleavage on fiber formation. Moreover, the effect of N-linked glycosylation on fiber formation could also be explored by a point mutation in the NIS sequence within the CT domain, as such sequence motif (NXS/T) is predicted for N-linked glycosylation (Mellquist *et al.*, 1998).

Summary (Paper III):

- The silk fusion protein Z-4RepCT was expressed and secreted using the methylotrophic yeast *P. pastoris* as heterologous host.
- Expression and secretion of Z-4RepCT were affected by parameters as temperature, pH and time.
- A mixture of full-length protein and degraded variants of Z-4RepCT could be purified from *P. pastoris* growth medium.

- A specific position in the CT domain subjected to cleavage was identified.
- The presence of both O-linked and N-linked glycosylation in expressed and secreted Z-4RepCT was determined.

2.2.4 Paper IV: Materials made from enzyme-silk fusion proteins show catalytic properties that are also retained after storage, reuse, cleaning with ethanol and in continuous substrate flow.

There are numerous reports on immobilization of enzymes using silk materials, although most of them concerns non-covalent immobilization strategies. However, covalent enzyme attachment to both silkworm silk (Zhu *et al.*, 2011; Saxena & Goswami, 2010; Chatterjee *et al.*, 2009; Vepari & Kaplan, 2006; Zhang *et al.*, 2005) and to recombinant spider silk (Spiess *et al.*, 2010; Huemmerich *et al.*, 2006) have been presented previously. In the two studies where recombinant spider silk was combined with enzyme, β-galactosidase was covalently attached to silk films using either carboxyl groups (Huemmerich *et al.*, 2006) or thiol groups (Spiess *et al.*, 2010) exposed on the film surface. As the principle of genetic fusion for covalent linkage of functional protein domains to recombinant 4RepCT silk was shown in paper II, the aim of this paper (paper IV) was to use the same genetic approach for linkage of an enzyme to 4RepCT silk, and also directly to the non-repetitive and conserved CT domain of 4RepCT. Further, the aim was to determine the catalytic ability of produced enzyme-silk materials in the form of fiber, surface coating and foam.

To explore the concept of enzyme-silk the choice of enzyme for genetic linkage fell upon endo-1,4-β-xylanase A (Paice *et al.*, 1986), a glycoside hydrolase derived from *Bacillus subtilis*. Xylanase was chosen since it is active in the monomeric form and since its activity is not dependent on glycosylation or disulfide bridges, thus suitable for recombinant production in *E. coli*. Moreover, the enzymatic activity of xylanase, to cleave internal 1,4-β-xylosidic bonds of heteroxylans, can easily be detected by colorimetric assays. Xylanases are also interesting from a commercial perspective, since they are frequently used in industry for various applications as bread making and paper manufacturing, and has also been investigated for conversion of biomass in biofuel production (Polizeli *et al.*, 2005; Saha, 2003; Courtin & Delcour, 2002; Beg *et al.*, 2001).

The two silk fusion proteins, Xyl-4RepCT and Xyl-CT, were successfully expressed in *E. coli* and subsequently recovered as soluble proteins after purification using IMAC. From SDS-PAGE analysis protein bands at expected molecular sizes were evident at 45 kDa and 34 kDa for Xyl-4RepCT and Xyl-CT, respectively. Protein identity of the silk fusion proteins were further

verified using mass spectrometry, identifying peptide fragments from both xylanase and the CT domain. However, both Xyl-4RepCT and Xyl-CT displayed an additional protein band of strong intensity positioned at around 30 kDa and 25 kDa on SDS-PAGE, respectively. Mass spectrometry analysis of these shorter protein versions revealed peptide fragments from xylanase, indicating the presence of full-length silk fusion protein and a truncated version thereof after purification of Xyl-4RepCT and Xyl-CT. Though, SDS-PAGE analysis revealed that the truncated protein variants were not incorporated into generated fibers, and thus cannot give any contribution to xylanase activity of the silk materials.

Both of the two soluble silk fusion proteins were able to self-assemble into macroscopic fibers, meaning that covalent linkage of the 20 kDa xylanase domain does not prevent the fiber forming abilities of the silk modules (4RepCT and CT), in spite of xylanase being as large as 4RepCT and almost twice the size of CT. Noticeable, however, was the decreased rate at which fibers were formed for Xyl-4RepCT and Xyl-CT compared to non-functionalized 4RepCT and to the silk fusion proteins presented in paper II. Soluble 4RepCT transforms into visible fibers within one hour, followed by a continuous fiber build-up for approximately five hours (Stark *et al.*, 2007). In contrast, the time for visible Xyl-4RepCT and Xyl-CT fibers to appear was prolonged to several hours with continuous fiber growth up to two weeks. The reason for this altered fiber formation rate is most probably a combination of the high solubility and the relatively large size of the covalently linked xylanase. The large size of xylanase may well decrease the possibility of the silk parts to interact and, thereby, prolong the time for fiber formation.

To test for enzyme activity, a colorimetric assay was used to determine the catalytic ability of xylanase covalently linked to the spider silk modules. Enzymatic activity was identified for soluble Xyl-4RepCT proteins, as well as for fibers made from soluble Xyl-4RepCT and Xyl-CT. These findings indicate that the xylanase domains have preserved their enzymatic capability when produced as xylanase-silk fusion proteins and more interestingly, even after further processing into xylanase-silk fibers.

With confirmed enzymatic activity for xylanase-silk fibers, production of other types of Xyl-4RepCT materials was explored in the form of surface coatings and foams. Two types of surface coatings were produced, one prepared from coating with soluble Xyl-4RepCT (denoted AdsCoat) and another prepared from a first coated layer of non-functionalized 4RepCT and a second layer of Xyl-4RepCT on top (denoted LAdsCoat). When assessed for xylanase activity it turned out that both coating types displayed activity, and at comparable levels.

The initially prepared Xyl-4RepCT foam did show enzymatic activity but suffered from poor stability, both from physical detachment of pieces of foam upon immersion in liquid, but also from release of soluble Xyl-4RepCT molecules from the foam. The issue of detachment of foam pieces during liquid immersion was solved by exposing the foam for elevated humidity in a steaming process, making the foam more flattened and thus, physically more stable. However, the problem with release of Xyl-4RepCT molecules during initial washing, as measured by the enzymatic activity in wash fractions, was still an issue for the steamed foam. In attempt to prevent also this problem, a third foam variant was produced; a mixed steamed foam. This foam was prepared as the former foam with the one exception that foaming was made from a mixture of soluble Xyl-4RepCT and 4RepCT. The mixed steamed foam displayed xylanase activity, with no material disintegration or removal of Xyl-4RepCT molecules. The overall increased stability for mixed steamed foam most likely stems from an increased probability of the silk modules to interact, due to mixing with non-functionalized 4RepCT. Noteworthy, the presented strategy for achieving stable xylanase-silk foam did not contain any treatment with alcohol, a method otherwise common in generating water-insoluble silk films, but also a potential mean of irreversibly denaturing attached fold-dependent protein domains.

Xylanase-silk materials were also investigated in terms of storage and reuse, the ability to withstand treatment with ethanol and sodium hydroxide, and the effect of steam sterilization. Concerning storage of xylanase-silk materials, it was shown possible to store both fibers and surface coatings without loss of enzyme activity. Interestingly, fibers could also be dried prior to storage and still show activity upon re-wetting, although with reduced activity. Reuse for at least three times, as well as the possibility of treatment with 70% ethanol, was shown for fiber and LAdsCoat. In contrast, treatment with sodium hydroxide and steam sterilization of fibers irreversibly destroyed the xylanase domain.

Two major advantages of having enzymes covalently attached to solid support are the possibility to reuse the enzymes and the ease of separation of products from enzymes. A processing method making use of both these benefits at the same time is continuous processing, in which the substrate is continuously flowed over the solid support with immobilized enzymes. Generated products from enzyme catalysis can then conveniently be collected in solution after passing the confined enzymes. To test the concept of continuous processing, a substrate solution was continuously pumped, and allowed to recirculate, through a column containing Xyl-4RepCT fibers. When the xylanase activity was measured for withdrawn fractions of substrate

solution allowed to pass the fibers for an increasing number of times, a gradual increase in activity was determined for up to twenty cycles. This implies that xylanase-silk fibers can be used also in continuous enzymatic processes, and not only in batch processes.

Summary (Paper IV):

- Two enzyme-silk fusion proteins with the enzyme xylanase genetically fused to 4RepCT and CT, respectively, could self-assemble into fibers.
- Fiber, surface coating and foam made from xylanase-silk fusion protein displayed enzymatic activity.
- The catalytic property was retained in xylanase-silk materials after storage, reuse and cleaning with ethanol, but not after sodium hydroxide treatment and steam sterilization.
- Xylanase-silk fibers displayed enzymatic activity in a continuous substrate flow.

3 Concluding remarks and future perspectives

Silk materials derived from *B. mori* silk fibroin and from recombinant spider silk proteins have shown great promise when investigated for its applicability in medicine and biotechnology. Characteristics as biocompatibility and biodegradability have encouraged the exploration of silk materials for use as scaffolds to support cells in culture as well as in tissue engineering. Other technological areas where silk materials have shown its potentiality are drug delivery, biosensors and electronics. Progress in formulation techniques allowing silk proteins to be shaped into materials of various morphologies has certainly contributed to broaden the range of applicability. Moreover, different approaches to functionalize silk have been used to equip silk materials with new functions most valuable in both medical and biotechnological applications. Extensive work has been conducted on silk fibroin due to the availability of the material, obtained in large quantities from cocoons of domesticized silkworms. However, technologies for recombinant protein production have in recent years allowed mimics of spider silk proteins to be produced in high amounts.

Coating of natural spider silk with various compounds has been shown feasible for production of functionalized silk fibers exhibiting electrical and fluorescence properties (Chu & Sun, 2007; Kim *et al.*, 2007). Using the same strategy, we investigated coating of recombinant spider silk (4RepCT) fibers with two polyelectrolytes (PEDOT-S and PTAA). Fibers stained with both types of polyelectrolytes were demonstrated and the properties of electrical conductivity (PEDOT-S) and fluorescence (PTAA) were induced. Furthermore, a set of parameters which highly affect the efficiency of staining were identified. The herein used 4RepCT silk have previously been shown suitable for cell culturing and, in addition, conducting polymers have been utilized for electrical stimulation of certain cell types (*e.g.*, muscle and nerve cells). In future studies, it would be interesting to investigate the effects of

conducting 4RepCT fibers, or other material formats thereof, on the electrical stimulation of such relevant cell types, especially if combined with functionalization with specific cell binding motifs and potentially even growth factors.

Genetic engineering has enabled the combination of recombinant spider silk sequences with other suitable genes, thereby obtaining functionalized spider silk fusion proteins. As an example, recombinant spider silk proteins have been genetically fused to cell-binding peptides (Widhe *et al.*, 2013; Wohlrab *et al.*, 2012; Bini *et al.*, 2006). Materials processed from these fusion proteins showed positive effects on cell adherence in cell cultures. In two of our studies we have investigated genetic functionalization of recombinant spider silk (4RepCT) with larger proteins domains, whose activity is of a more complex nature. In the first of these studies, fusion to protein domains (Z, C2, ABD, M4) with affinity for IgG, albumin and biotin, respectively, were explored. Produced silk fusion proteins were successfully processed into silk-like materials displaying fold-dependent affinity towards their intended targets. In the second study, recombinant spider silk was functionalized with enzymatic activity by genetic linkage to an enzyme. After processing of enzyme-silk fusion proteins into fibers, coatings and foams, enzymatic activity was observed in all formats. These findings show upon the possibility of genetic functionalization of recombinant spider silk with active proteins domains, resulting in silk fusion proteins with retained ability to form silk-like materials and preserved functionality of added domains.

To show upon a more general applicability for biomolecule presentation, the IgG-binding fusion protein Z-4RepCT were used to decorate films with antibodies directed against the growth factor VEGF. Molecules of VEGF were in this way effectively captured onto the film via the bound anti-VEGF antibodies. As growth factors play an important role in advanced cell culturing, presentation of growth factors, or other relevant biomolecules, achieved by capture onto Z-4RepCT or C2-4RepCT materials decorated with relevant antibodies, could be useful for advanced cell culturing in the future. It would also be interesting to investigate antibody decorated silk materials for utilization in biosensors for detection of a target molecule or even simultaneous detection of a mixture of target molecules. Moreover, the concept of functionalizing spider silk with enzyme could also be utilized in biosensors. The herein demonstrated genetic functionalization technique could have an impact on the sensitivity of detection, thanks to the possibility of high density provided by that the active domain can be fused to every silk molecule.

Other expression hosts than *E. coli* could be advantageous in order to enhance the production of recombinant spider silk fusion proteins. The

methylotrophic yeast *P. pastoris* has been widely used as a heterologous host for recombinant protein production, mainly because of high titers of expressed proteins and the possibility for protein secretion. Although *E. coli* was used as host for expression of most constructs in this thesis, *P. pastoris* was also investigated as a host for expression and secretion of Z-4RepCT proteins. It was demonstrated that Z-4RepCT could be expressed and secreted by *P. pastoris*, although purified fractions contained a mixture of full-length and degraded proteins. A position in the CT domain of 4RepCT subjected to cleavage was identified and it was also revealed that the CT domain was subjected to both N-linked and O-linked glycosylation. The non-successful self-assembly into silk-like fibers of Z-4RepCT purified from *P. pastoris* was postulated to originate from the observed alterations in the CT domain. To get around the problem of cleavage, point mutations at the observed cleavage site could be performed. Likewise, to investigate the impact of glycosylation on self-assembly, a set of point mutations at the identified potential glycosylation sites in the CT domain could also be performed.

Taken together, the results presented in this thesis show upon physical and genetic functionalization of recombinant spider silk for induction of electrical, affinity and enzymatic properties. Such properties could find their potential use in biosensor systems as well as in advanced cell culture applications.

4 Populärvetenskaplig sammanfattning

Under århundraden har silke fångat människans uppmärksamhet på grund av dess mekaniska egenskaper som ett av de starkaste naturliga material vi känner till. Sedan ett tiotal år tillbaka har silkesmaterial även rönt ett stort intresse för användning inom mer avancerade medicinska och bioteknologiska områden. Materialegenskaper hos silke såsom biokompatibilitet (hur väl ett material tolereras av kroppen), biodegraderbarhet (i vilken utsträckning ett material kan brytas ned i kroppen) och mekaniska egenskaper (hur fysiskt starkt ett material är) har visat sig särskilt lämpade för medicinska tillämpningar och är starkt bidragande orsaker till det ökande intresset för silkesmaterial. Det har även genomförts en hel del studier på silkesbaserade material relaterade till nämnda tillämpningsområden, dock har de allra flesta av dessa studier koncentraters till en specifik typ av silke, nämligen silke från silkesmask. En av anledningarna till detta är att det är jämförelsevis lätt att få tag på stora mängder av detta material, eftersom det kan utvinnas från silkesmaskens spunna kokonger. Tillgänglighet är en viktig aspekt om man vill studera olika tillämpningar av ett material på bred front.

En annan vanligt förkommande typ av silke är spindeltrådssilke, ”spindeltråd”, som används av spindlar för konstruktion av sina nät. Spindlar är experter på att spinna olika typer av trådar, vissa starkare än andra, och ett spindelnät består vanligtvis av ett antal olika trådtyper med olika uppgifter. Spindeltråd har samma goda materialegenskaper, i vissa aspekter till och med bättre, som silkesmasksilke. Detta har gjort att spindetråd också har ansetts vara ett bra kandidatmaterial för medicinska tillämpningar. Problemet hittills har dock varit att det inte är särskilt lätt att få tag i tillräckligt stora mängder av materialet för att kunna bedriva forskning i någon större utsträckning. Att låta spindlar tillverka spindeltråd som man sedan skall ta tillvara är ett väldig ineffektivt sätt att arbeta, främst på grund av den lilla mängd tråd som man kan erhålla från varje enskild spindel. Detta läge har drastiskt förändrats i och med

utvecklingen av genteknik, med vilken man kan manipulera andra organismer än spindeln själv att producera varianter av de komponenter (proteiner) som utgör spindeltråd. Dessa proteinvarianter utgör sedan basen för ”konstgjorda spindeltrådsmaterial”. En idag vanligt förekommande organism för detta ändamål är tarmbakterien *Escherichia coli* (*E. coli*) som har använts för att tillverka olika varianter av spindeltrådproteiner. Värt att notera i sammanhanget är att de riktiga spindeltrådsproteinerna är generellt sätt för stora för att kunna tillverkas av bakterier, varför man har plockat ut eller designat lagom stora bitar som fortfarande innehar originalproteinernas unika egenskaper.

För att fullt ut kunna utnyttja silkesmaterialens potential inom avancerad medicin eller annan teknologi skulle man vilja ha möjligheten att tillföra nya egenskaper och funktioner till dessa material, en process kallad ”funktionalisering”. Detta har gjorts i en hel del studier på silkesmasksilke, men även på konstgjorda spindeltrådsmaterial, där man till exempel har funktionaliserat materialet med enzymer, modellmolekyler för läkemedel samt olika molekyler med effekt på odling av humana celler. Med möjligheten att kombinera de unika egenskaperna hos silkesmaterial med nya egenskaper, följer också möjligheten att skräddarsy funktionaliserade silkesbaserade material för tillämpning inom områden såsom avancerad medicin eller bioteknik. Mot bakgrund av detta har denna avhandling fokuserat på strategier för att funktionalisera ett specifikt konstgjort spindeltrådsmaterial som används i vår forskargrupp. Byggstenarna i detta silkesmaterial består av ett protein kallat 4RepCT, som är en del av ett protein som bygger upp en av de starkaste spindeltrådarna man känner till och som tillverkas av spindeln *Euprosthenops australis*.

I en av avhandlingens artiklar (artikel I) tillverkades först fibrer (trådar) från 4RepCT-proteiner. Fibrerna gjordes sedan elektriskt ledande genom att färgas in med den elektriskt ledande polymeren PEDOT-S. Vi observerade att parametrar som temperatur och surhetsgrad påverkade hur effektivt fibrerna färgades. Denna typ av funktionalisering är icke-kovalent vilket betyder att de två materialen (fibrerna och polymeren) inte är sammanlänkade med kemiska (kovalenta) bindningar, utan istället attraheras till varandra av icke-kovalenta bindingar såsom elektrostatiska (laddningsberoende) interaktioner. Funktionalisering genom infärgning är en enkel metod genom att man i princip bara kan doppa fibrerna i en lösning innehållande ämnet som man vill färga in med. För att visa att denna metod även kan användas för att färga in fibrer med andra molekyler funktionaliserades 4RepCT-fibrer även med polymeren PTAA för att på så sätt erhålla fluorescerande fibrer. I studier som har gjorts av andra forskargrupper har man kunna visa att elektrisk stimulans av vissa celltyper

(t.ex. nervceller och muskelceller) kan ha positiv effekt på utvecklingen av dessa celler. Av den anledningen vore det intressant testa sådana celleffekter med våra elektriskt ledande 4RepCT-fibrer.

Begreppet genetisk sammanlänkning betyder här att man på DNA-nivå kopplar ihop genen for 4RepCT med en gen för ett annat protein. På så sätt tillverkas ett så kallat fusionsprotein från produktion i till exempel *E. coli*. Fusionsproteinet kommer ha både silkesegenskaper och egenskaper från det proteinet som man har kopplat det till. Vidare så är dessa två hoplänkade med en stabil kovalent bindning. Vi har visat att man kan använda genetisk sammanlänkning för att funktionalisera 4RepCT med affinitetsegenskaper (artikel II) och mer komplexa enzymegenskap (artikel IV). I båda dessa studier observerade vi att förmågan hos 4RepCT-delen av fusionsproteinerna att kunna spontanbilda fibrer fortfarande finns kvar även när 4RepCT sitter ihop med andra proteindomäner. Vi såg också att egenskaperna hos de proteindomäner som sammanlänkades med 4RepCT fortfarande fanns kvar i silkesmaterial producerade från respektive fusionsprotein. För att visa på möjligheten att binda in mer avancerade biomolekyler till funktionaliserade silkesmaterial användes fusionsproteinet Z-4RepCT, där Z-proteindomänen kan binda till antikroppar, för att dekorera Z-4RepCT-filmer med tillväxtfaktorer via antikropar som först bundits till Z-4RepCT. Att kunna binda biomolekyler, som till exempel är mycket viktiga för odling av humana celler, är värdefullt för avancerade cellodlingstillämpningar.

I artikel III utvärderades jäst som en alternativ värdorganism till *E. coli* för produktion av fusionsproteinet Z-4RepcT. Jästarten *Pichia pastoris* valdes i denna studie på grund av möjligheten till produktion av stora mängder fusionsprotein samt det välkända faktum att producerade proteiner kan transporteras ut ur jästcellerna. Vi kunde visa att det var möjligt att producera Z-4RepCT-proteinet i jästen och att proteinet dessutom kunde transporteras ut ur jästcellerna. Dock fann vi att proteinet hade klyvts av på minst ett ställe samt att proteinet hade modifierats genom glykosylering (sockermolekyler som är kovalent fastsatta på proteinet).

Sammanfattning:

- Två olika strategier för att införa nya egenskaper (funktionalisera) hos material baserade på det spindeltrådsinspirerade 4RepCT-proteinet har demonstrerats, infärgning och genetisk sammanlänkning.
- Genom infärgning av 4RepCT-fibrer med två olika polymerer kunde fibrer med elektriskt ledande och fluorescenta egenskaper genereras.
- Genetisk sammanlänkning användes för att på DNA-nivå koppla ihop 4RepCT med proteindomäner med affinitet för molekylerna IgG, albumin

och biotin. De producerade 4RepCT-fusionsproteinerna kunde spontant bilda fibrer med bibehållen förmåga hos proteindomänerna att binda sina respektive målmolekyler.

- Genetisk sammanlänkning användes även för att utrusta 4RepCT med en mer komplex funktionalitet i form av enzymaktivitet. Alla olika producerade materialtyper uppvisade enzymatisk aktivitet.
- Vi visade att det är möjligt att producera ett 4RepCT-fusionsprotein i jästen *P. pastoris*, men att nedbrytning och glykosylering av proteinet kan vara ett problem för spontan fiberbildning.

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