Functionalization of partial spider silk with affinity domains and its use for diverse applications

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Cover: Representation of functionalized partial spider silks (RC and NC) for use in diverse applications.

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Abstract

Over the past years, spider silk has drawn considerable attention from researchers because of its renowned mechanical strength (force needed to break), elasticity, biocompatibility and biodegradability. The advancements in genetic engineering have led to the production of artificial mimics of spider silk proteins.

The main objective of this thesis is to functionalize two variants of partial spider silk, 4RepCT (RC), 23 kDa and NTCT (NC), 27 kDa, by covalent attachment to affinity domains at gene level. Retained functional properties of the silk part and added affinity domains were studied in the resulting silk fusion proteins.

In Paper I, four affinity domains of different sizes (5-17 kDa) with different folds were genetically attached to the RC silk variant. We confirmed that all four RC silk fusion proteins could self-assemble to silk-like fibers. The ability of each added affinity domain to bind its respective target while in RC silk fusion protein was also confirmed. A non-covalent way of presenting biotinylated growth factors was achieved by one of the constructs; M4-4RepCT silk. In a similar way, presentation of an active enzyme was verified by the activity measurement of the silk-fusion material with bound enzyme. Thus, these findings highlight the use of such materials in for example cell culture and tissue engineering applications.

In Paper II, as a proof-of-concept, two recombinant antibody fragments (scFvs) each of 30 kDa, previously shown to contribute to the candidate protein signature for diagnosing systemic lupus erythematosus (SLE), were covalently attached to either ends of RC and NC silk variants. All of the generated scFv-RC and scFv-NC silk fusion proteins were shown able to self-assemble to fibres. The retained functionalities of scFv domains in scFv-RC/NC silk fusion proteins were confirmed in micro- and nanoarrays, respectively. Significantly higher target detection signal was reported by scFv-silk fusion proteins when compared to the same added amount of scFvs alone in the immunoassays. Thereby, suggesting the use of scFv-silk fusion proteins as capture probes in generation of sensitive diagnostic immunoassays.

The overall results from this thesis thus highlight the diverse possible applications of partial spider silk proteins after being functionalized with various affinity domains.

Keywords: affinity domains, biotinylated ligands, immunoassays, partial spider silk, single chain variable fragments, functionalization.

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Dedication

To my parents and friends

May all be happy; May all be without disease; May all have well-being; May none have misery of any sort.

-Brihadaranyaka Upanishad 1.4.14

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

II **Thatikonda N**., Delfani P., Jansson R., Petersson L., Lindberg, D., Wingren C., Hedhammar M. (2016). Genetic fusion of single-chain variable fragments to partial spider silk improves target detection in micro- and nanoarrays. Biotechnology Journal 11 (3), 437-448.

The contribution of Naresh Thatikonda to the papers included in this thesis was as follows:

I Planned the studies, performed laboratory work and analyzed data, for the work related to the fourth construct. Participated in the discussions related to other constructs together with minor contribution to writing the manuscript.

II Planned the studies, performed majority of the work related to the construction, expression and purification of all recombinant silk constructs employed in the study. Analyzed results together with major contribution to writing the manuscript.

Abbreviations

ABD	Albumin binding domain
B. mori	*Bombyx mori*
CT	C-terminal domain
E. australis	*Euprosthenops australis*
E. coli	*Escherichia coli*
EGF	Epidermal Growth Factor
ECM	Extracellular Matrix
IMAC	Immobilized Metal ion Affinity Chromatography
MaSp	Major ampullate spidroin
NT	N-terminal domain
N. clavipes	*Nephila clavipes*
scFv	Single chain variable fragment
VEGF	Vascular Endothelial Growth Factor

1 Introduction

The capability to generate silk, a natural protein fiber, has developed by spiders, several arthropods and 23 groups of insects (Walker *et al.*, 2012; Sutherland *et al.*, 2010; Altman *et al.*, 2003). Due to the remarkable mechanical and elastic properties of the silk derived from spiders, combined with its biocompatibility, spider silk has been exploited for biomaterial and biomedical applications (Borkner *et al.*, 2014; Bittencourt *et al.*, 2012; Widhe *et al.*, 2012; Omenetto & Kaplan, 2010). However, additional functionalization is needed for generation of more advanced materials, which otherwise does not exist in nature. Processing of silk from cocoons produced by silkworms require harsh processing steps involving for example, degumming in alkali solution followed by solubilization in organic solvents like hexafluoroisopropanol, which restrict their use in applications where additional functionalization is needed (Rockwood *et al.*, 2011). The work described in this thesis is mainly based on the silk derived from spider silk, thus information regarding other kinds of silk is not described and can be found elsewhere (Kasoju & Bora, 2012; Sutherland *et al.*, 2010).

1.1 Overview of spider silk

1.1.1 Natural spider silk

In nature, orb weaving spiders can generate up to seven types of silks, which have different physical properties and perform different functions (Rising *et al.*, 2011). Out of the seven types of spider silks, dragline silk that is produced in the major ampullate gland of spider has widely been studied for its incredible mechanical properties (Borkner *et al.*, 2014; An *et al.*, 2012; Bittencourt *et al.*, 2012). Spider dragline silk is composed of two high molecular weight proteins (Ayoub *et al.*, 2007; Bini *et al.*, 2006), namely Major ampullate spidroin 1 and 2 (MaSp1 and MaSp2, respectively) (Humenik *et al.*, 2011). The dragline silk spidroins produced by various species of spiders

share a common tripartite protein architecture (Humenik *et al.*, 2011). They are composed of a non-repetitive N-terminal domain, a repetitive region mainly containing poly-alanine segments interspersed between glycine rich repeats, and a non-repetitive C-terminal domain (Ayoub *et al.*, 2007). The repetitive region, which is composed of different amino acid segments, was reported to account for the amazing physical properties of the dragline silk (Rising *et al.*, 2011). Naturally, spiders produce silk in liquid form (dope) and store it at high concentrations (50% w/v) within specialized silk glands (Chen *et al.*, 2002). Upon changes in the ion composition, pH, dehydration (Walker *et al.*, 2012), and shear forces occurring along the duct (Dicko *et al.*, 2004; Knight & Vollrath, 2001); stored silk solution will emerge out as silk fibers, which are rich in β-sheets (Gosline *et al.*, 1999; Simmons *et al.*, 1996). The intermolecular interactions occurring between the poly-alanine segments and glycine rich repeats in the repeat region (Rising *et al.*, 2011), combined with the N-terminal (Askarieh *et al.*, 2010) and C-terminal domain (Hagn *et al.*, 2010) of the spidroins were reported to regulate the process of silk fibre formation.

The dragline silk of *Euprosthenops* is stronger than that from other species of spiders like *Nephila*, *Araneus* and *Latrodectus* (Stark *et al.*, 2007; Pouchkina-Stantcheva & McQueen-Mason, 2004). Advantages and limitations in production of natural and engineered dragline silks are addressed in a later section.

1.1.2 Recombinant production of partial spider silk in bacteria

The predatory behaviour of spiders together with the insufficient amounts of silk produced by spiders, have restricted the option to perform sericulture of spiders and hence opted for recombinant production of spider silk proteins (Guhrs *et al.*, 2000). Recombinant production is defined as the expression of a foreign gene in a production host, for example bacteria (Tokareva *et al.*, 2013). Due to the well characterized genome and low costs involved in production, *Escherichia coli* (*E.coli*) is often considered as a suitable option for large scale protein production.

Due to the repetitive nature and large size of natural spider silk genes in general, issues like premature translational stops, low protein yield and homologous recombination to host genome, can be of concern when expressed in the bacteria (Arcidiacono *et al.*, 1998). For this reason, partial spider silk sequences are preferred for production of artificial spider silk mimics in bacteria (Tokareva *et al.*, 2013; Stark *et al.*, 2007; Prince *et al.*, 1995). However, with recent progress in the field of genetic engineering together with the modular nature of spider silk, different partial spider silk proteins derived

from different species of spiders have been produced in various hosts including yeast, bacteria, insects, plants and mammalian cells with varying success (Tokareva *et al.*, 2013; Rising *et al.*, 2011). Some of the partial spider silks which were cloned and expressed in bacteria include repeat segments derived from MaSp1 and MaSp2 of *Nephila clavipes* with (Prince *et al.*, 1995) or without (Mello *et al.*, 2004) inclusion of a native C-terminal domain. Another example of partial spider silk is 4RepCT (RC silk), composed of four poly-alanine/glycine rich co-segments together with a C-terminal domain derived from MaSp1 of *Euprosthenops australis* (*E. australis*) (Stark *et al.*, 2007). Likewise, another partial spider silk variant, NTCT (NC silk) was herein generated by combining native N-terminal and C-terminal domains derived from *E. australis*. The study presented in this thesis is based on these two partial spider silk variants, RC and NC silks.

Major steps involved in the recombinant production of partial spider silk proteins in bacteria are (Figure 1): (i) Isolation of full length spider silk cDNA from spider, (ii) Selection of cDNA that codes for partial spider silk protein, (iii) restriction cleavage of target vector, (iv) ligation of partial spider silk cDNA to the cleaved target vector, (v) transformation of generated recombinant DNA (rDNA) into bacteria, (vi) expression of rDNA in the bacteria, (vii) harvesting the purified partial spider silk proteins from the bacteria mainly using affinity purification (Stark *et al.*, 2007; Prince *et al.*, 1995). All the steps are represented in Figure 1.

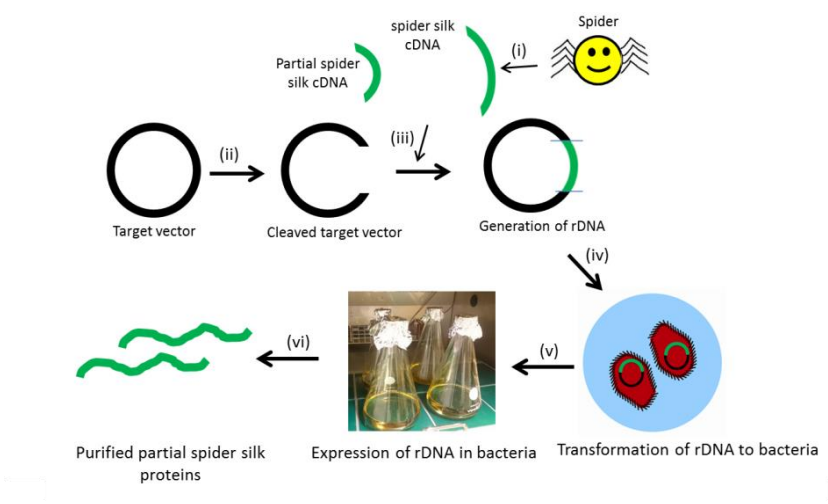

Figure 1. Scheme of recombinant production of partial spider silk proteins in bacteria.

1.1.3 Processing of partial spider silk proteins to various formats

The success of recombinant production in bacteria has enabled a convenient way to produce partial spider silk proteins under denaturing (Humenik *et al.*, 2011) and non-denaturing conditions (Stark *et al.*, 2007). Moreover, the produced partial spider silk proteins have an advantage of being possible to process into unnatural silk morphologies like, foam, film and mesh formats with (Hardy & Scheibel, 2009) or without (Widhe *et al.*, 2010) being subjected to post-treatment with organic solvents for β-sheet enrichment. Partial spider silk variants which are produced under non-denaturing conditions without any requirement of organic solvents during material fabrication might have better opportunities for modification with a wide range of biologically active molecules.

1.1.4 Modification of partial spider silk proteins

Chemical or genetic modification of partial spider silk proteins will broaden the applicability window of silk based materials (Wohlrab *et al.*, 2012; Spieß *et al.*, 2010). Chemical modification can be achieved by the use of established procedures that permit efficient and site-specific modifications of proteins (Hackenberger & Schwarzer, 2008). However, certain coupling chemicals used for modification of silk materials might exhibit side-effects that are detrimental to cells when used for tissue engineering applications (Wohlrab *et al.*, 2012). In those cases, genetic modification of partial spider silk proteins could be used as a more beneficial alternative. The possibility to genetically modify partial spider silk has been shown by the addition of cell binding motifs using genetic engineering method with convincing results in cell culture studies (Widhe *et al.*, 2016; Widhe *et al.*, 2013; Wohlrab *et al.*, 2012).

1.2 Different ways to immobilize proteins onto solid supports

Methods which rely on the binding of target molecules to complementary "bait" molecules depend on the immobilization of those bait molecules to a solid support. This section describes different ways to immobilize proteins, which constitute one important group of such bait molecules. For example, antibodies are immobilized to solid support for their use in affinity purification and immunoassays.

Immobilization of proteins on solid surfaces is of great importance in the production of protein based arrays and biosensor related applications. The challenging task is to immobilize the proteins in such way their functionality and orientation are maintained, and also to remain their conformation unaltered after immobilization (Oshige *et al.*, 2013). Furthermore, restricting the

interaction between the immobilized antibodies and the solid surface might help to achieve an appreciable portion of antibodies that retain their native confirmation.

Addressing the above challenges can allow for the successful use of immobilized proteins for their intended applications. The chemical and structural complexity of proteins have created difficulties in determining a general strategy for immobilization of proteins (Saerens *et al.*, 2008). Different ways to immobilize proteins on various surfaces have been reported, namely 1) non-covalent physical adsorption, 2) covalent coupling via free amines or thiol groups and 3) non-covalent protein linkage via affinity interactions (*e.g.*, His_6/Ni-NTA) (Oshige *et al.*, 2013; Kimple *et al.*, 2010). In all cases, physical and chemical properties of the surface and also other factors like temperature and pH have been shown to affect the surface immobilization of the proteins (Oshige *et al.*, 2013).

Non-specific physical adsorption is a quick way to immobilize protein molecules, but often leads to protein denaturation (Butler *et al.*, 1993). In order to prevent the denaturation of immobilized proteins, they can be spotted onto solid surfaces, which have been pre-coated with e.g. polyacrylamide gels or polyethylene glycol (Pollak *et al.*, 1980). In the covalent coupling method, reactive functional groups present in the protein molecules (*e.g.*, amines, thiols) are crosslinked onto the solid support modified with complementary reactive groups, thus ensuring protein immobilization (Oshige *et al.*, 2013; Camarero, 2008). However, the immobilization methods described above does often not result in well oriented protein molecules, which is necessary for efficient binding of their target molecules (Oshige *et al.*, 2013; Camarero, 2008). The orientation of immobilized protein molecules can be improved by use of a non-covalent protein immobilization via affinity. For example, proteins carrying histidine tags can be immobilized onto solid surface which is coated with Ni-NTA molecules. However, use of non-covalent means of immobilization is limited by the lack of long-term stability of the immobilized proteins (Camarero, 2008).

1.3 Affinity domains

One important class of proteins that is beneficial to have immobilized onto surfaces for certain applications are affinity domains.

1.3.1 Domains derived from bacterial surface receptors

In the recent past, exploiting the host defense mechanisms developed by bacteria for their use in the field of biotechnology has gained considerable

attention (Diamandis & Christopoulos, 1991). Bacterial strains of the *Staphylococci*, *Streptococci* and *Streptomyces* genera express certain proteins in order to evade the host immune system (Sauer-Eriksson *et al.*, 1995; Diamandis & Christopoulos, 1991). Out of the several IgG-binding bacterial proteins that were identified, staphylococcal protein A (SPA) and streptococcal protein G (SPG) are extensively studied for their use in the purification of antibodies (Boström *et al.*, 2012).

SPA contains five homologous IgG-binding domains. The Z domain (7 kDa), which folds into a three-helix bundle, is an engineered protein domain derived from the IgG-binding B-domain of SPA (58 kDa) (Boström *et al.*, 2012; Nilsson *et al.*, 1987). SPG has a multi domain structure containing both albumin-binding domains and immunoglobulin-binding domains (Kraulis *et al.*, 1996). The C2 domain (6 kDa), which folds into four β-sheets and α-helix structure is derived from the Fc binding domain B1 of SPG (Sauer-Eriksson *et al.*, 1995). Both Z and C2 domains can be used for the purification of antibodies or target proteins fused to the Fc domain. SPA and SPG exhibit different specificities to IgG obtained from different species (Boström *et al.*, 2012). Likewise, species specific IgG binding of the domains derived from SPA and SPG can be speculated.

The ABD domain is an engineered protein domain derived from albumin-binding domain 3 of SPG (63 kDa). Domain ABD (5 kDa) has affinity towards human serum albumin and folds into a three-helix bundle (Nilvebrant & Hober, 2013; Kraulis *et al.*, 1996).

1.3.2 Domain derived from streptavidin

Streptavidin is a protein produced by *Streptomyces avidinii*, which has affinity towards biotin and biotinylated ligands (Diamandis & Christopoulos, 1991). The extreme affinity between biotin and streptavidin (Diamandis & Christopoulos, 1991) complicate the release of biotinylated ligands (Rybak *et al.*, 2004). This resulted in the generation of monomeric streptavidin (M4) (Wu & Wong, 2005), which exhibit a lower binding affinity than streptavidin, in order to expand the applicability of biotin/streptavidin system for several biotechnological applications. M4 is a 16.5 kDa monomeric version of streptavidin, which has affinity to biotin molecules and folds into a β-sheet barrel structure (Wu & Wong, 2005).

1.3.3 Domains derived from antibodies

Naturally, antibodies are produced by B-cells in order to protect against foreign substances. As represented in Figure 2, antibodies are composed of two heavy and two light chains joined together by disulfide bonds. There are three

constant domains and one variable domain in the heavy chains, while there is one constant domain and one variable domain in the light chains (Figure 2). The epitope recognition site of an antibody is in the variable heavy (VH) and variable light (VL) domains. Due to excellent target specificity and affinity, antibodies are considered as paradigm for cell biology and biochemistry applications (Gebauer & Skerra, 2009). However, structural complexity and laborious procedures involved in the production of antibodies have led to creation of recombinant antibody fragments such as scFv and Fab (Gebauer & Skerra, 2009) (Figure 2). Considerable loss of biological activity has been observed upon immobilization of antibodies onto solid supports, which affects the sensitivity of the antibody based immunoassays, for example. Furthermore, the presence of the Fc part of the antibody can result in unwanted cell reactivity upon binding to the effector cells containing Fc receptors (Holliger & Hudson, 2005).

Generation of antibody mimics was considered as a breakthrough in the field of antibody engineering. Monovalent Fabs (55 kDa) are generated from full-length antibodies subjected to papain cleavage (Porter, 1959), whereas, single-chain variable fragment, scFv (28 kDa), one of the most popular formats of antibody derivatives, is generated by the fusion of variable domains of the heavy and light chain (VH and VL) joined by a flexible linker. Generated Fab and scFv fragments are smaller in size when compared to the antibodies (Figure 2) and can be expressed in *E. coli* with retained antigen binding affinity of the intact IgG (Bird *et al.*, 1988). Due to the smaller size of scFv and due to less efficient folding and assembly of Fab fragments in *E. coli* (Hust *et al.*, 2007), scFv is considered as a suitable affinity ligand for different applications, immunoassays for example. The biomolecular properties, like stability, can be enhanced by engineering scFvs (Worn & Pluckthun, 2001).

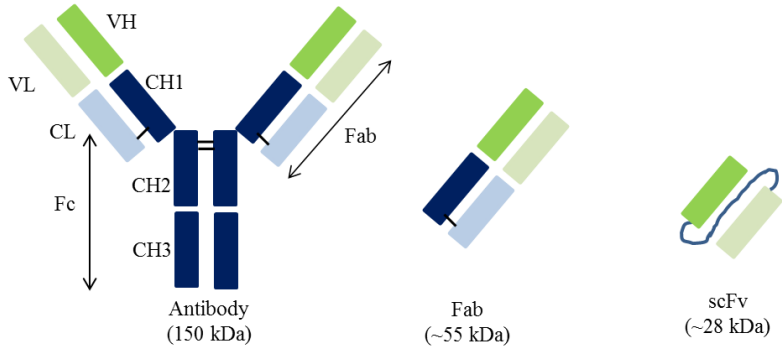

Figure 2. Representation of an antibody and domains derived from an antibody.

1.4 Diverse applications of functionalized partial spider silk

The relevance of functionalized partial spider silk for diverse applications is an actively growing field of research. Partial spider silk proteins, which can be produced using recombinant DNA technology can be subjected to genetic modifications by incorporation of peptides, recognition motifs or functional domains (Borkner *et al.*, 2014). In the recent past, cell binding peptides have successfully been incorporated to different engineered spider silk proteins, thereby suggesting the applicability of such functional silk materials for *in vitro* cell culture studies (Widhe *et al.*, 2013; Wohlrab *et al.*, 2012). Furthermore, silver binding peptides (Currie *et al.*, 2011) and antimicrobial peptides (Gomes *et al.*, 2011) were genetically incorporated to partial spider silk and the added peptides were reported to retain their functionalities, thus highlighting the use of silk-silver and silk-antimicrobial materials for biomedical applications. Likewise, uranium recognition motifs were genetically fused to a partial spider silk protein derived from *N. clavapies*, and then the uranium binding ability of the chimeric silk-uranyl proteins were confirmed. This highlights the applicability of such functional silk fusion proteins as biosensor capture probe for environmental monitoring applications (Krishnaji & Kaplan, 2013).

Affinity domains are complex and larger in size when compared to the peptides or recognition motifs. Therefore, exploring the possibility to incorporate affinity domains to partial spider silk might increase the applicability of functional silk based materials for other applications, for example immunoassays, advanced biosensor and cell culture applications.

2 Present investigations

2.1 Aims of the thesis

The main aim of the present thesis is to immobilize different affinity domains to two variants of partial spider silk proteins (RC and NC) via genetic engineering technique. Moreover, to study the functional properties of the silk part and added affinity domains in RC/NC silk fusion proteins. Furthermore, explore the applicability of such functional RC/NC silk fusion proteins for different applications. The specific aims of this thesis have been:

- **Paper I**: To investigate the possibility to functionalize RC silk proteins with different fold-dependent affinity protein domains using genetic fusion/linkage followed by functional studies of silk materials generated from the RC silk fusion proteins.

- **Paper II**: To covalently combine two scFvs to RC and NC silk protein variants at the gene level and to evaluate the possibility to use scFv-RC/NC silk fusion proteins as capture probes for generation of sensitive immunoassays.

2.2 Results and discussion

2.2.1 Paper I: Recombinant spider silk genetically functionalized with affinity domains.

In paper I, functionalization of RC silk with various affinity domains was evaluated. Four different fold dependent affinity domains (Z, C2, ABD and M4) were covalently attached to the N-terminus of 4RepCT at the gene level.

Domains Z (7 kDa) (Nilsson *et al.*, 1987) and ABD (5 kDa) (Kraulis *et al.*, 1996) are folded into a three helix bundle, and bind to IgG and albumin respectively. In contrast, C2 (6 kDa) and M4 (17 kDa) domains contain β/α domains (Sauer-Eriksson *et al.*, 1995) and β-sheet barrel (Wu & Wong, 2005) respectively, and have affinity towards IgG and biotin, respectively. All four recombinant RC silk constructs were successfully produced in the BL21 (DE3) strain of *E. coli* and purified using immobilized metal ion affinity chromatography under non-denaturing conditions. It is worth noting that the produced RC silk fusion proteins could still be self-assembled to film and fiber format despite the relatively large sizes (5-17 kDa) of the affinity domains added to the RC silk. Moreover, no additional processing steps are required to make fibers and films of RC silk fusion proteins compared to RC silk alone. Since target binding ability of the added affinity domains is dependent on their secondary structure, Fourier transform infrared spectroscopy (FTIR) analysis was performed for films and fibers made from all RC silk fusion proteins. FTIR analysis supported maintained β-sheet structure, which is a characteristic of 4RepCT, in both film and fiber formats of RC silk fusion proteins. In order to evaluate the selective IgG binding ability of Z and C2 domains, a complex serum mixture was added to the fibers and films of Z-4RepCT and C2-4RepCT. Bound IgG was then released and run on the SDS-PAGE, which showed a band corresponding to the correct size of IgG (140 kDa), thus confirming retained functionality of added Z and C2 domains to selectively bind IgG. Likewise, the functionality of added ABD was verified upon incubation of complex human plasma to fiber and film of ABD-4RepCT followed by the release of bound albumin and gel analysis that confirmed the maintained functionality of ABD to selectively bind albumin.

In order to explore the possibility to create multi-functional silk materials, mixed films and mixed fibers were prepared by combining soluble fractions of Z-4RepCT and ABD-4RepCT. The ability of mixed films and fibers to selectively capture both IgG (by Z-4RepCT) and albumin (by ABD-4RepCT) was shown by addition of human plasma to the mixed silk materials followed by the release of both bound target molecules as shown by gel electrophoresis, thus confirming the concept of multi-functional silk materials.

Due to the simplicity and versatility of the biotin-streptavidin system, it has gained a significant focus in the field of biotechnology (Diamandis & Christopoulos, 1991) and also for being used to link functional groups to biomaterials (Wang & Kaplan, 2011; Spieß *et al.*, 2010). Likewise, in the quest to link functional groups to RC silk based materials, the possibility to covalently link the monomeric streptavidin domain (M4) to 4RepCT has been verified in **Paper I**. To demonstrate the biotin binding ability of the M4 domain in the M4-4RepCT construct, films of M4-4RepCT were incubated with biotinylated DNA molecules. High affinity between monomeric streptavidin and biotinylated ligands (Wu & Wong, 2005) resulted in a difficulty to release the bound biotinylated ligands. Since release of bound biotinylated ligands usually requires harsh elution conditions (Rybak *et al.*, 2004), a restriction enzyme site was included at the 5' end of the biotinylated DNA molecules to ensure mild release, which could be confirmed by a DNA gel. Thus the applicability of M4-4RepCT silk materials for nucleic acid hybridization assays is envisioned. Furthermore, functionalization of M4-4RepCT films with growth factors was evaluated. This concept was confirmed by capture of EGF molecules pre-labeled with a chromophoric biotin onto M4-4RepCT films, followed by measurement of the absorbance signal that come from the bound biotinylated EGF molecules.

In a recent study, a recombinant silk construct named FN-4RepCT was shown to enhance the cell supportive ability of spider silk (Widhe *et al.*, 2016). Upon combining soluble FN-4RepCT and M4-4RepCT, an advanced silk based cell scaffold could possibly be designed for *in vitro* cell culture studies, provided that the basal culture medium is supplemented with biotinylated growth factors. Moreover, due to the high affinity between M4 domain and biotinylated ligands (Wu & Wong, 2005), long term use of bound biotinylated growth factors is envisioned during media change, thus providing an economic benefit.

On the other hand, if release of growth factors is demanded by the cells and if it is difficult to biotinylate growth factors, a reversible two-step presentation of growth factor via Z-4RepCT materials could instead be employed. The feasibility of such two-step approach using Z-4RepCT silk was shown by the capture of the growth factor vascular endothelial growth factor (VEGF) using anti-VEGF IgG, which was first bound to the Z domain. To visualize the indirect binding of VEGF to Z-4RepCT films via anti-VEGF IgG, the VEGF was biotinylated and a streptavidin labelled with fluorophore was used for detection.

Since it is challenging to determine the function of biotinylated growth factors captured to 4RepCT fusions, a domain with more easily measured

functionality, the enzyme xylanase, was biotinylated and captured onto M4-4RepCT films. Activity of bound xylanase was thereafter verified upon conversion of the substrate to a product that could be measured at a certain wavelength. Higher absorbance signal was observed from M4-4RepCT films with biotinylated xylanase than corresponding Z-4RepCT control films, thus confirming the maintained activity of the xylanase. This finding highlights the possible use of M4-4RepCT silk materials to capture other enzymes which have potential applications in the field of biology and medicine (Costa *et al.*, 2005).

2.2.2 Paper II: Genetic fusion of single-chain variable fragments to partial spider silk improves target detection in micro- and nanoarrays.

In paper II, one of the attractive features of spider dragline silk proteins, namely stickiness (Vollrath, 2000) has been exploited for the development of high performing recombinant antibody based immunoassays. In this study, two single chain variable fragments (scFvs), α_{VEGF} and α_{C1q} (30 kDa) that bind to the serum proteins VEGF (low abundant) and C1q (high abundant) respectively, were selected from a human recombinant scFv phage-display library (Soderlind *et al.*, 2000). Corresponding genes were then genetically attached to either ends of two partial spider silk variants (RC and NC). All of the generated RC/NC silk fusion constructs (RC-α and α-RC, 52 kDa, NC-α and α-NC, 54 kDa) could be efficiently produced in *E. coli* and purified using immobilized metal ion affinity chromatography (IMAC). Even though a partial degradation of target proteins was observed, scFv-silk fusion proteins of sufficient quality were obtained. Concentrated fractions of soluble scFv-RC/NC silk fusion proteins could be processed to silk like fibers. This confirmed a retained functionality of RC and NC silk to form silk like fibers despite when covalently attached to scFv domain (30 kDa), which is larger in size compared to the sizes of both RC (23 kDa) and NC (27 kDa) silk proteins.

To investigate the target binding affinities of added scFv domains in scFv-RC silk constructs, a microarray slide carrying microspots of scFv-RC silk fusion proteins (RC-α_{VEGF} & α_{VEGF}-RC, RC-α_{C1q} & α_{C1q}-RC) was designed. Since silk protein provides a hydrophilic environment and mechanical stability to the array spot, this might change the morphology of the array spot and thereby affect the outcome of immunoassay. Therefore, mixed spot (soluble scFvs and RC added together) and double spot (soluble scFv added onto dried RC) formats were formulated to constitute controls of scFvs non-covalently attached to RC silk. Incubation of VEGF antigens onto a microarray slide revealed VEGF binding to the microspots containing α_{VEGF}, RC-α_{VEGF} and α_{VEGF}-RC proteins but almost no binding to the other microspots. Similarly,

addition of C1q antigens to the microarray slide showed binding of C1q antigens only to the microspots of α_{C1q}, RC- α_{C1q} and α_{C1q}-RC. These observations also confirmed that there exists no cross reactivity among different scFv-RC silk fusion proteins. Moreover, target detection signal was distinctly increased when scFvs were covalently attached to RC silk compared to the same added amount of scFvs to the microspots containing only scFv. The increase in target binding signal might be due to the high propensity of spider silk proteins to self-assemble and adhere to the solid surfaces, thereby resulting in well oriented antibody fragments from the fusion protein. Presence of well oriented immobilized antibody fragments would enable efficient binding to the target molecules, thereby contributing to the success of solid-phase assays. Likewise, in another study, use of well oriented antibody fragments has been shown to improve the target binding (Hu *et al.*, 2013). Another reason for improved target detection signal might be that a higher proportion of scFvs might have stayed within the array spots during the several processing steps involved in the immunoassay when attached to the silk protein. On the contrary, very low target detection signal, close to the background signal, was obtained from mixed and double spots, thus demonstrating the necessity of having scFvs covalently linked to silk when used as capture probes in immunoassays. Incubation of non-fractionated serum sample onto the microarray slide containing microspots of scFv-RC silk fusion proteins confirmed the selective binding between the scFv-silk fusion proteins and their respective targets present in the serum. This highlights the ability of scFv-RC silk fusion proteins to bind their targets even from a complex sample.

In the quest for generation of sensitive and high density arrays, possible use of scFv-silk fusion proteins as the capture probes was investigated also using nanoarrays. Deposition of proteins for nanoarrays can be done by several techniques, for example using dip-pen technology (Petersson *et al.*, 2014; Wingren & Borrebaeck, 2007). The repetitive region in the RC silk variant has a high tendency to fibrillate, which could potentially clog the nozzle if deposited using dip-pen technology. To avoid this problem, NTCT (NC), a new engineered variant of spider silk was generated, without including the repetitive region, and used for nanoarray analysis. Again the previously selected scFvs (α_{VEGF} and α_{C1q}) were covalently attached onto either ends, resulting in the generation of scFv-NC silk fusion proteins (NC-α_{VEGF} & α_{VEGF}-NC, NC-α_{C1q} & α_{C1q}-NC). Similar to microarray analysis, upon addition of pure antigen samples (VEGF and C1q) onto the nanoarray slides containing nanospots of scFv-NC silk fusion proteins, no sign of cross reactivity was observed among different scFv-NC silk fusion proteins. Furthermore, significantly higher target detection signal was observed by scFvs when

covalently attached to NC silk compared to the same added amount of scFvs alone. Thus, the results from the nanoarray analysis were in line with microarray results.

In order to rule out unspecific binding of serum components to the silk, C1q depleted serum was prepared. Upon addition of C1q depleted serum onto a scFv-silk coated nanoarray slide, very low target binding signal was reported from NC-α_{C1q} & α_{C1q}-NC, confirming low unspecific binding of serum components to scFv-NC silk proteins. To study the array setup using a clinically relevant sample, pooled serum from Systemic Lupus Erythematosus (SLE) patients was examined. Upon incubation of serum from Systemic Lupus Erythematosus (SLE) patients onto the nanoarray slide, a higher target signal was reported by α_{VEGF}-NC silk fusion protein compared to incubation with C1q depleted serum. This might be due to the high levels of VEGF in serum derived from SLE patients (Zhou *et al.*, 2014), which is an hallmark of patients with SLE disease compared to low levels of VEGF present in the serum obtained from normal patients. The results from the nanoarray analysis confirmed a retained functionality of the two scFvs that were attached to NC silk.

To summarize, in **Paper II** a procedure to immobilize antibody fragments via partial spider silk, which would result in well oriented antibody fragments, was demonstrated. Moreover use of scFvs covalently attached to the N-terminus of partial spider silk could yield stable scFvs within array spots. Thus future use of such *in vitro* targeting agents for sensitive diagnostics is envisioned.

3 Summary

Technological advancements have led to the generation of artificial mimics of spider silk proteins in amounts sufficient for their use in certain applications. Characteristics like biocompatibility and biodegradability exhibited by partial spider silk signify their use for cell culture and tissue engineering applications.

The capability of partial spider silk to adhere to the surfaces can be considered for generation of sensitive immunoassays and biosensor related applications.

In **paper I**, by use of genetic engineering, RC silk was functionalized with fold dependent affinity domains (Z, C2, ABD and M4) of different sizes (5-17 kDa). The resulted RC silk fusion proteins were shown to retain the ability of the silk part to self-assemble into silk like fibers. Moreover, the target binding ability of the added affinity domains was retained. RC silk functionalized with the biotin-binding M4 domain opens up the opportunity to use such functional silk materials for several different applications. Ability of M4-4RepCT to bind biotinylated growth factors has been validated in **paper I**. Thus, M4-4RepCT silk materials carrying immobilized growth factors could be used as extracellular matrix (ECM) analogues for cell culture applications. In many cases, attachment of biotin to macromolecules does not alter the biological activity of the macromolecules (Diamandis & Christopoulos, 1991). Procedures to biotinylate various macromolecules are well established, enabling a wide repertoire of available biotinylated macromolecules. Capture of such biotinylated macromolecules by M4-4RepCT silk materials could be a convenient way to generate various functional silk materials, which could have implications for diverse applications.

In **paper II**, functionalization of RC and NC silk variants was carried out by the covalent attachment of two recombinant antibody fragments (α_{VEGF} and α_{C1q}, 30 kDa) to both silk variants at the gene level. Results from this study demonstrate the scalability in the size of affinity domains that can be covalently attached to the partial spider silk. The functionality of recombinant

antibody fragments in the produced scFv-RC/NC silk fusion proteins was confirmed by immunoassays. Specific and significantly improved binding of the scFv-silk fusion proteins to their respective targets was detected. Furthermore, no cross reactivity between different scFv-silk fusion proteins was observed. Thus, a novel procedure to immobilize scFvs via partial spider silk has been described. The applicability of such improved probe format in generation of sensitive immunoassays for efficient disease diagnosis is thus envisioned.

In summary, the ability of partial spider silk to be furnished with different functional domains expands the application horizon of such functional partial spider silk materials.

4 Concluding remarks

Paper I: Affinity domains that are cloned into recombinant spider silk retain their binding specificity when the fusion protein is coated onto a solid surface and processed to fiber format. This means that the affinity domain part of the fusion protein is folded in such a way that binding properties are maintained, and that it is exposed on the surface of the silk well enough to enable efficient binding of its target.

Paper II: By genetic linkage of antibody fragments into recombinant silk, it is possible to improve sensitivity and signal strength of immunoassays where the silk fusion protein is coated onto a surface, as compared to coatings of the antibody fragments alone.

5 Future studies

Paper I:

- Fibers produced from recombinant spider silk were previously reported to be well tolerated when implanted in rats (Fredriksson *et al.*, 2009). Likewise, in future, fibers generated from M4-4RepCT silk could be first used to capture biotinylated antimicrobial drugs for example, amikacin (Boyce *et al.*, 1993) followed by their implantation to the wounded site. Such type of materials carrying antimicrobial drugs could likely be used for local and sustained delivery of drugs to alleviate wound healing.
- Ability of Z-4RepCT silk to capture growth factors in a two-step procedure was shown in **paper I**. Possibility to use Z-4RepCT silk with captured growth factors as *in vitro* cell culture scaffold should be evaluated.

Paper II:

- The surface properties of the array slide were shown to affect the functionality of immobilized biomolecules (Seurynck-Servoss *et al.*, 2007). Therefore, future study of herein used scFv-silk fusion proteins on various array slides made of different materials could provide better understanding for their efficient use as capture probes in sensitive diagnostic applications.
- Two recombinant antibody fragments (α_{VEGF} and α_{C1q}) used in **paper II** were selected from a collection of scFvs that contribute to a candidate protein signature for diagnosing the autoimmune disease SLE (Petersson *et al.*, 2014; Carlsson *et al.*, 2011). In future investigations, covalent attachment of other scFvs to the N-terminus of NC silk variant could be performed for generation of a sensitive nanoarray slide to diagnose SLE disease.
- Collection of scFv fragments that contribute to the candidate protein signature for diagnosis of breast (Olsson *et al.*, 2013) and pancreatic

cancers (Wingren *et al.*, 2012) are available. Future use of herein described silk based scFv immobilization strategy can possibly result in the generation of sensitive array chips for efficient diagnosis of prostate and breast cancers.

- Shelf life study of the array slides containing capture probes is an important parameter to consider when designing a chip for disease diagnosis. Therefore, long term shelf life studies of the array slides containing scFv-silk fusion proteins should be evaluated.
- The ability of α_{VEGF}-RC silk coating to bind VEGF protein was verified in **paper II**. In future, solutions of the cell adhesion promoting FN-silk (Widhe *et al.*, 2016) and VEGF growth factor binding α_{VEGF}-RC silk can be mixed and processed to combined silk coat. Future study of cellular behavior on cell culture plates containing such combined silk coatings would provide valuable information for generation of artificial cell culture matrices.
- Anti-VEGF based therapies has been developed to inhibit the growth of new blood vessels, thereby starving tumor cells (Ellis & Hicklin, 2008). Since α_{VEGF}-RC silk can bind to VEGF, the possibility of α_{VEGF}-RC silk to bind VEGF in a way which prevents the angiogenesis process could be investigated. Exploring this possibility enables the use of α_{VEGF}-RC as a biopharmaceutical for treatment of certain cancers.

6 Popular scientific summary

Over several decades spider silk has widely been known for its incredible mechanical properties in terms of toughness, strength and elasticity. Spider silk is reported to be stronger and tougher than steel and high performance synthetic fibers, like Kevlar, by weight. In addition to amazing mechanical properties, biocompatibility and biodegradability exhibited by spider silk fibers has made spider silk as the subject of research for different biomedical and material applications. Certain spiders, like orb weaving spiders, can produce several types of silk from various silk glands and the produced silks differ in their primary amino acid sequence, physical properties and functions. Out of several types of silk produced by spiders, dragline silk, which is used by the spiders to capture their prey, is the strongest. Even though researchers have succeeded in deciphering the valuable secrets shared by spiders, still there exist no possible way to produce spider silk in commercial quantities. The cannibalistic behavior exhibited by spiders restricted their farming. A great deal of effort by material scientists and biologists had resulted in generation of partial spider silk protein in amounts sufficient for certain biomedical applications. Furthermore, compared to synthetic high-performance materials, artificial spider silk is produced using renewable resources. If produced at large scale, spider silk might has the ability to be used as an alternative to stainless steel, thus contribute to the generation of ecological materials.

Mainly for economic reasons, *E. coli* bacteria, has often been chosen for the production of partial spider silk. Since large gene size of full length spider silk cannot be handled by bacteria, pieces of full length spider silk gene are selected and were transferred to bacteria thus resulted in the production of partial spider silk proteins.

The applicability of produced partial spider silk proteins for various applications within the field of medicine and biotechnology can be increased by a process called functionalization, in which different biological properties are incorporated to partial spider silk. Likewise, this thesis is focused on

functionalization of two partial spider silks (4RepCT (RC) and NTCT (NC)) with various affinity domains using genetic engineering. RC and NC partial spider silks are derived from the dragline silk of the spider *E. australis*. In **paper I** and **II**, we have shown that different affinity properties can be incorporated to RC and NC partial spider silks by covalently combining various affinity proteins to partial spider silk proteins using genetic engineering techniques, thus resulting in RC and NC silk fusion proteins. Both in paper **I** and **II,** we observed that the ability of the silk part in RC and NC silk fusion proteins to spontaneously form fibers is still maintained despite the fusion of different protein domains. Moreover, the target binding properties of the added protein domains are maintained in the RC and NC silk fusion proteins. In paper **I,** we could show the ability of M4-4RepCT and Z-4RepCT silk materials to bind biomolecules in two different ways, which are essential for human cell culture. We also showed the ability of M4-4RepCT to present an active enzyme in **paper I**. Thus use of such silk materials has been contemplated for their use in tissue engineering applications. In paper **II**, we have shown that the ability of anti-VEGF and anti-C1q proteins to recognize their target molecules has significantly been improved when attached to NC silk. This hints the future of NC silk fusion proteins for applications where improvement of target recognition is required.

7 Populärvetenskaplig sammanfattning

Under flera decennier har spindeltråd varit allmänt känd för sina otroliga mekaniska egenskaper i termer av bland annat styrka och elasticitet. Spindeltråd rapporteras vara starkare än stål och högpresterande syntetfibrer, såsom Kevlar, per vikt. Förutom fantastiska mekaniska egenskaper har även biokompatibilitet och bionedbrytbarhet hos spindeltrådsfibrer gjort spindeltråd intressant för olika biomedicin- och materialtillämpningar. Vissa spindlar kan producera flera olika typer av silke från olika silkeskörtlar och de framställda silkena skiljer sig åt i sin primära aminosyrasekvens, fysikaliska egenskaper och funktioner. Av de olika typerna av silke som produceras av spindlar är ”dragline-silke”, som används av spindlar för att fånga sitt byte, den starkaste. Även om forskare har lyckats dechiffrera värdefulla hemligheter om spindlar och dess tråd finns det fortfarande inget sätt att framställa spindeltråd i kommersiella kvantiteter. Det kannibalistiska beteende som spindlar uppvisar har begränsat möjligheten att föda upp spindlar i större skala. Gemensamma arbetsinsatser av materialforskare och biologer har dock resulterat i generering av partiella silkesproteiner i mängder som är tillräckliga för vissa biomedicinska tillämpningar. Dessutom är artificiell spindeltråd producerad med hjälp av förnybara resurser jämfört med syntetiska material. När de produceras i stor skala skulle spindelsilke kanske ha förmåga att kunna användas som ett alternativ till rostfritt stål i vissa applikationer och på så sätt bidra till genereringen av ekologiska material.

Av främst ekonomiska skäl har ofta *E. coli*-bakterier använts för framställning av partiellt spindelsilke. Eftersom den stora genstorleken av naturliga spindeltrådsproteiner inte kan hanteras av bakterier har istället kortare bitar av fullängdsgenen valts ut och överförts till bakterier för att på så sätt producera partiella spindelsilkesproteiner istället.

Användbarheten av de producerade partiella silkesproteinerna för olika tillämpningar inom områden som medicin och bioteknik kan ökas med en process som kallas funktionalisering, där olika biologiska egenskaper

inkorporeras i det partiella spindelsilket. Denna avhandling fokuserar på funktionalisering av två partiella spindelsilkesproteiner (4RepCT (RC) och NTCT (NC)) med olika affinitetsdomäner med hjälp av genteknik. Partiellt RC- och NC-silke härstammar från dragline-silke från spindeln E. australis. I **artikel I** och **II** har vi visat att olika affinitetsegenskaper kan tillföras RC- och NC-silke genom att kovalent kombinera olika affinitetsproteiner med de partiella silkesproteinerna genom att använda genteknik, vilket resulterar i RC- och NC-silkesfusionsproteiner. Både i **artikel I** och **II** observerade vi att förmågan hos silkesdelen i RC- och NC-fusionsproteinerna att spontant bilda fibrer fortfarande upprätthålls trots kovalent koppling till de olika affinitetsdomänerna. Dessutom bibehölls de målmolekylbindande egenskaperna hos de tillsatta affinitetsdomänerna i RC- och NC-fusionsproteinerna. I **artikel I** kunde vi demonstrera förmågan hos M4-4RepCT- och Z-4RepCT-silkesmaterial att binda biomolekyler på två olika sätt, båda väsentliga för till exempel odling av humana celler. Vi visade också på förmågan hos M4-4RepCT att presentera ett aktivt enzym i artikel I. Vi tror att sådana här funktionaliserade silkesmaterial skulle kunna användas för avancerade cellodlingstillämpningar, såsom i fältet ”tissue engineering”. I **artikel II** visade vi att förmågan hos anti-VEGF- och anti-C1q-proteiner för att känna igen sina målmolekyler väsentligt förbättrades när de var kovalent kopplade till NC-silke. Detta antyder att NC-silkesfusionsproteiner i framtiden kan användas för applikationer där det krävs en förbättring av målmolekyligenkänning.

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