Artificial Spider Silk

Recombinant Production and Determinants for Fiber Formation

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2008

Acta Universitatis agriculturae Sueciae
2008:100

Cover: Female *Euprosthenops australis* (photo: A. Rising).

ISSN 1652-6880
ISBN 978-91-861-9533-5

Tryck: SLU Service/Repro, Uppsala 2008

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Abstract

Spider dragline silk is Nature's high-performance fiber that outperforms the best man-made materials by displaying extraordinary mechanical properties. In addition, spider silk is biocompatible and biodegradable, which makes it suitable as a model for biomaterial production. Dragline silk consists of large structural proteins (spidroins) comprising an extensive region of poly-alanine/glycine-rich tandem repeats, located in between two non-repetitive and folded terminal domains. The spidroins are stored at high concentration in liquid form and are converted to a solid fiber through a poorly understood spinning process. In order to artificially replicate the dragline properties, the protein constituents must be characterized and the silk production pathway elucidated. The large, repetitive sequences of the genes and corresponding proteins have made spidroin analogs difficult to produce in recombinant expression systems. Genetic instability, prematurely terminated synthesis and poor solubility of produced proteins are often observed.

This thesis presents a novel method for the efficient recombinant production of a soluble miniaturized spidroin under non-denaturing conditions. The mini-spidroin can be processed under physiological conditions to form fibers with favorable mechanical and cell-compatibility properties, without the use of denaturing spinning procedures or coagulation treatments. The fibers structure and macroscopic appearance resemble native spider silk and the strength equals that of regenerated silk and mammalian tendons. In addition, for the first time, the production of recombinant silk with enhanced mechanical properties was accomplished through introduction of mutations, enabling covalent intermolecular cross-linking of proteins constituting the fiber. Moreover, the effect on structure, stability and fiber forming propensities for all representative parts of MaSp1, due to changes in temperature, pH and salt concentrations was investigated.

Keywords: Recombinant expression, silk, Major ampullate spidroin, dragline, biocompatible, biodegradable, disulphide bond, protein structure.

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To my family

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 Grip S., Rising A., Nimmervoll H., Storckenfeldt E., McQueen-Mason S. J., Pouchkina-Stantcheva N., Vollrath F., Engström W., Fernandez-Arias A. (2006). Transient expression of a major ampullate spidroin 1 gene fragment from *Euprosthenops* sp. in mammalian cells. *Cancer Genomics & Proteomics* 3, 83-88.

II Stark M., Grip S.*, Rising A.*, Hedhammar M., Engström W., Hjälm G., Johansson J. (2007). Macroscopic fibers self-assembled from recombinant miniature spider silk proteins. *Biomacromolecules* 8, 1695-1701.* Equal contribution.

III Hedhammar M., Rising A., Grip S., Martinez A. S., Nordling K., Casals C., Stark M., Johansson J. (2008). Structural properties of recombinant nonrepetitive and repetitive parts of major ampullate spidroin 1 from *Euprosthenops australis*: implications for fiber formation. *Biochemistry* 47, 3407-3417.

IV Grip S., Johansson J., Hedhammar M. Engineered disulfides improve mechanical properties of recombinant spider silk (submitted).

Abbreviations

Three and one letter codes for the 20 naturally occurring amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Organisms

A. diadematus	*Araneus diadematus*
A. aurantia	*Argiope aurantia*
B. mori	*Bombyx mori*
E. australis	*Euprosthenops australis*
L. hesperus	*Latrodectus hesperus*
N. clavipes	*Nephila clavipes*
N. madagascariensis	*Nephila madagascariensis*
N. senegalensis	*Nephila senegalensis*
P. pastoris	*Pichia pastoris*

Other abbreviations

ADF	Araneus Diadematus Fibroin
AFM	Atomic Force Microscopy
CD	Circular Dichroism
FTIR	Fourier Transform Infrared
gpd	Grams per denier
HFIP	Hexafluoroisopropanol
kDa	Kilodalton
kb	Kilobases
MaSp	Major ampullate spidroin
MiSp	Minor ampullate spidroin
NMR	Nuclear Magnetic Resonance
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
Spidroin	Spider silk fibroin (protein)
TEM	Transmission Electron Microscopy

1 Introduction

Spider silk has fascinated man through all times and has been recognized for its remarkable properties. Spiders can produce up to seven different silks with different properties and functions, with the dragline silk being one of the strongest (Gosline et al., 1986). Spider dragline silk is Nature's high-performance biopolymer and is tougher than the best synthetic materials made by man (Gosline et al., 1999). Techniques for manufacturing stockings and gloves from spider cocoon silk were described as early as in the beginning of the 18th century (Bon, 1710-1712) but webs have also found its use as fishing nets, and single fibers as cross hairs in optical instruments (Vollrath, 1992). There are historical records of spider silk webs being used in early folklore medicine all over the world, and have for example been applied to open wounds in order to stop hemorrhage and promote wound healing (Newman and Newman, 1995). It is thought that spider webs have good clotting properties due to the fine size of its threads and that it also could have bactericidal properties (Vollrath et al., 2002, Vollrath et al., 1990, Vollrath and Knight, 2001). In order to function as a potential biomaterial, biocompatibility is obviously an important prerequisite, and *in vivo* studies have shown that spider silk is biodegradable and evoke a comparable defense reaction as materials routinely used in surgery (Vollrath et al., 2002, Gellynck et al., 2008).

In contrast to many of its synthetic counterparts, dragline silk is produced at ambient temperature and pressure using renewable resources and water as solvent. Despite vast efforts, synthetic materials with mechanical properties matching the dragline silk have not been produced. Understanding how the spider processes the proteins constituting the silk and structure-function relationships have been in the interest of researchers in recent years. These topics are important in the pursuit of copying the spider's complex spinning

machinery, and in the end, mimic the extraordinary fiber. Unfolding this Nature's mystery could enable the manufacturing of lightweight high performance materials for uses ranging from tough, energy absorbing applications such as parachutes and bullet proof vests, to scaffold devises in tissue regeneration. Achieving this could revolutionize the industry of material production that would provide an environmental friendly manufacturing process and product, contrary to what is available today.

1.1 Silks

The word silk is most often associated with the filaments produced by the domesticated silkworm *Bombyx mori*, but the fact is that a number of organisms produce a variety of silks with different properties and for varying purposes (Craig, 1997, Denny, 1980). For example, bees embed simple silk fibers into the wax of their combs to provide strength (Hepburn, 1988, Sutherland et al., 2007), and aquatic midge larvae use silk to construct tubes for feeding, housing and pupation (Smith et al., 1995, Wellman and Case, 1989).

Silk has been defined as "fibrous proteins containing highly repetitive sequences of amino acids that are stored in the animal as a liquid and configure into fiber when sheared or "spun" at secretion" (Craig, 1997). Silks are exclusively produced by the arthropods and only by animals in the classes Insecta, Arachnida and Myriapoda (Craig, 1997), but contrary to other silk-producing organisms, spiders are able to produce, and are dependent on silk throughout their lifespan. Spiders evolutionary success is tightly linked to the development of specialized glands producing silks with differing functions (Lucas, 1964, Gosline et al., 1986, Vollrath, 1992), which are used in the most elaborate ways. This is best displayed by the orb-web weaving spiders that use silk in nearly every aspect of their life.

Orb-web weaving is characteristic of species in two lineages of spiders: Araneoidea and Deinopoidea (Garb et al., 2006), with the former being the most extensively studied. The araneoids can produce up to seven different silks (Gosline et al., 1986, Kovoor, 1987, Hu et al., 2006) used in the elaborate aerial catching nets (Blackwall, 1830, Witt and Reed, 1965) along with other essential silk-based constructions, as displayed in Table 1.

Silks from orb-web weaving spiders are produced in opisthosomal (abdominal) glands (Figure 1) that through a secretory duct connects to modified setae called spigots, which in turn are located on reduced abdominal appendages, the spinnerets (Kovoor, 1987, Shear et al., 1989). The first records of silk production from opisthosomal spigots, and therefore spiders, are about 385 million years old (Shear et al., 1989).

Table 1. *Silks of orb-web weaving spiders*

Gland	Function	Core fiber proteins
Major ampullate (dragline)	Web frame, safety line	MaSp1[1], MaSp2[1]
Minor ampullate	Web reinforcement and temporary capture silk	MiSp1[2], MiSp2[2]
Flagelliform	Capture spiral	Flag[3]
Aciniform	Wrapping silk, small diameter egg case silk fiber	AcSp1[4]
Tubuliform	Large diameter egg case silk fiber	TuSp1[5], ECP-1[6], ECP-2[6]
Aggregate	Glue coating for capture spiral	SCP-1[7], SCP-2[7]
Pyriform	Attachment disk and joining fibers	Unknown

Adapted from Hu, *et al.* (2006). Data on aggregate silk from Hu, *et al.* (2007).
[1]Major Ampullate Spidroin 1 and 2, [2]Minor ampullate Spidroin 1 and 2, [3]Flagelliform silk protein, [4]Aciniform Spidroin 1, [5]Tubuliform Spidroin 1, [6]Egg Case Protein 1 and 2, [7]Spider Coating Peptide 1 and 2.

By disclosing the predominant building blocks found in the silk as amino acids, it was in 1907 suggested that silks are protein-based biopolymer filaments, see (Lewis, 1992). In the second half of the 20:th century research intensified with studies on physical, mechanical and chemical properties of spider silks (Warwicker, 1960, Lucas, 1964, Denny, 1980, Gosline et al., 1984, Lucas et al., 1960, Tillinghast et al., 1987, Tillinghast et al., 1981, Andersen, 1970). This initial research evoked a broad interest for these protein-based, high performance polymers, which is still growing.

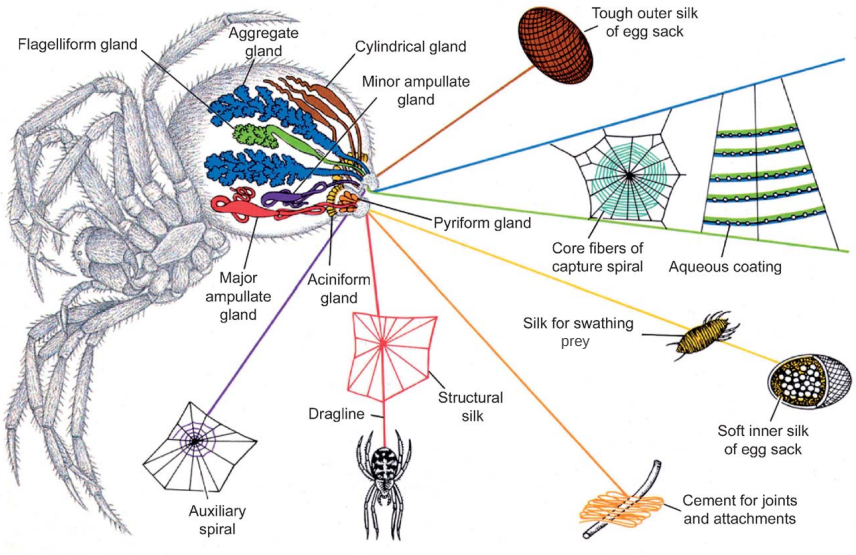

Figure 1. Silk gland distribution and corresponding silks produced by a typical orb-web weaving spider. Within its abdomen, the spider produces up to seven different silks in different glands, all used for different purposes. Modified from Dicko *et al.* (2006).

The dragline made by orb-web weaving spiders is one of the most extensively studied silks because of its extreme and unique mechanical properties. The combination of high tensile strength together with considerable extension capabilities allows the dragline to absorb a lot of kinetic energy before it breaks, and in this respect it outperforms the best man-made materials such as high-tensile steel and Kevlar (Gosline et al., 2002, Gosline et al., 1999, Swanson et al., 2006).

1.2 The major ampullate gland

The major ampullate gland of orb-web weaving spiders that produce the dragline silk can be divided into three parts according to morphology and function: the tail, the sac, and the duct (Figure 2). The overall morphology of the major ampullate gland is also consistent with that found in cob-weaving spiders (Casem et al., 2002). The proteins constituting the fiber are mainly produced by the tail, stored in the gland and structured in the duct (Bell and Peakall, 1969, Peakall, 1969).

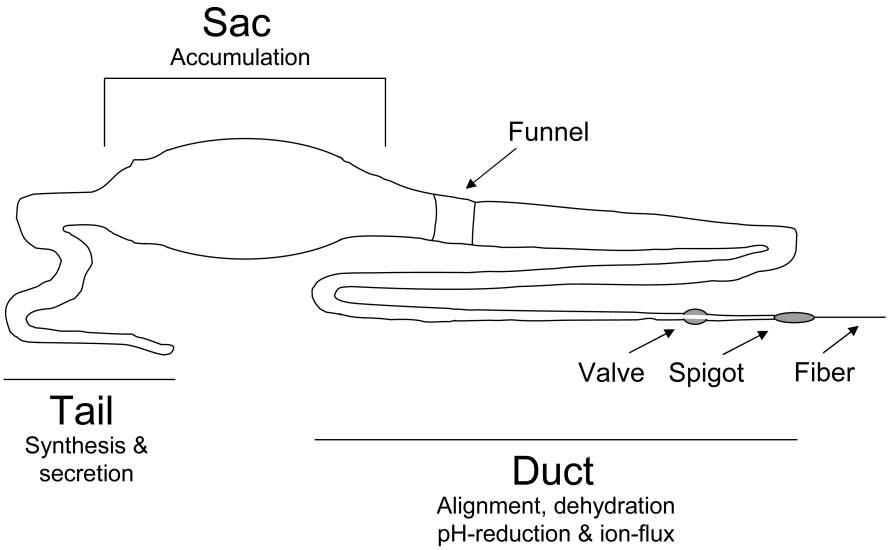

Figure 2. A schematic representation of the major ampullate gland and its sections with associated functions. The major ampullate gland is composed of three distinct parts; the tail, the sac and the duct with its three limbs folded into an S-shape. The funnel represents the end of the sac and the beginning of the duct. The valve is located in the end of the third limb of the duct and is followed by the spigot from which the silk fiber is extruded. The picture is not drawn to scale.

The bulk of the silk protein synthesis occurs in the tail of the gland. The epithelium of the tail presents a simple columnar arrangement, consisting of a single type of secretory cell. These cells appear to be specialized for the production of large amounts of protein. They have well-developed rough endoplasmic reticulum and are rich in a single type of secretory granule that accumulate in the apical region of the cells before being discharged into the lumen of the gland (Bell and Peakall, 1969, Plazaola and Candelas, 1991, Casem et al., 2002).

The wall of the sac is composed of a single layer of epithelial cells resembling those found in the tail. However, the volume of the secretory epithelial cells found in the tail is ten times of those lining the sac, and the protein synthesis of the sac is only 25% of that of the tail. This means that only a few percent of the total amount of silk protein produced by the gland is attributed the sac. Therefore, together with the fact that the volume of the sac lumen is twice of that of the tail, the main function of the sac is believed to be storage of protein dope (Peakall, 1969).

Dragline silk proteins mainly consist of alanine and glycine (Andersen, 1970, Lucas et al., 1960, Lombardi and Kaplan, 1990), and in glands mechanically stimulated to produce silk proteins (Candelas and Cintron, 1981), a selective increase in tRNAs corresponding to the predominant amino acids in the silk was observed. Moreover, a gland specific alanine-tRNA was discovered, which implies that the glands are provided with a functionally adapted tRNA population that allow optimal decoding of the gland's silk protein templates (Candelas, 1990).

The sac ends in a truncated conical structure, sometimes referred to as the funnel that marks the start of the gradually narrowing duct (Knight and Vollrath, 1999). The duct is folded into three limbs and is five times longer than necessary in order to connect the sac to the spinnerets (Bell and Peakall, 1969, Sponner et al., 2005b, Vollrath and Knight, 1999). The duct is lined internally by a cuticular intima produced by epithelial cells (Kovoor, 1987) and connects to a spigot on the base of one of the anterior spinnerets (Wilson, 1969). The duct was early postulated to be involved in the retraction of water from the protein dope during fiber formation and to have a role in orientation of the silk proteins (Bell and Peakall, 1969, Tillinghast et al., 1984). This has been corroborated by later studies with cryo-SEM of unfixed glandular material were it was found that the cuticle that lines the glands duct had the structure of an advanced hollow fiber dialysis membrane thought to facilitate a rapid removal of water and/or change in ionic composition involved in the spinning process (Vollrath and Knight, 1999). The observation of morphological characteristics such as desmosomes, closely packed microvilli and infolding of the basal membrane in the epithelial cells of the distal part of the duct further suggest a specialization for ion and water transport (Vollrath et al., 1998, Bell and Peakall, 1969, Casem et al., 2002). Intra-abdominal pressure is believed to ensure that the duct is constantly filled with protein dope accessible for instantaneous silk spinning. There are no muscles around the soft-walled gland, which could directly force the fluid silk down the duct, so it has therefore been suggested that this function is performed by the internal hydrostatic pressure (Wilson, 1962a).

The valve, an anatomical feature seen as a thickening of the cuticular lining of the duct, is situated near the base of the anterior spinneret (Wilson, 1962b). The valve, with associated muscles, has been suggested to function as a brake-clamp controlling the spiders decent on its dragline (Wilson, 1962b, Vollrath and Knight, 1999). In addition, based on anatomical studies,

the valve has been proposed to assist in restarting spinning after internal rupture of a thread (Vollrath and Knight, 1999).

1.3 The structural proteins of the dragline

The dragline silk is composed of at least two large structural proteins, often termed major ampullate spidroins (MaSp1 and MaSp2) or fibroins (ADF-3 and ADF-4). The nomenclature depends on from which spider species the dragline originates. ADF-4 corresponds to MaSp1 and ADF-3 to MaSp2 (Xu and Lewis, 1990, Hinman and Lewis, 1992, Guerette et al., 1996, Sponner et al., 2005a). It is suggested that one of the proteins (spidroin 1) represents the majority of the two spidroins (Brooks et al., 2005, Hinman and Lewis, 1992), and it was observed that the proteins are unevenly represented in the silk dope and dragline (Sponner et al., 2005b, Sponner et al., 2005a).

Even though distinct primary structure variations exist between the two dragline spidroins, and between dragline spidroins from different spider species, they all have a highly conserved general architecture in common (Gatesy et al., 2001) (Figure 3).

Figure 3. Schematic picture of the general architecture of dragline silk proteins. The non-repetitive N- and C-terminal domains are depicted in green. Blue boxes represent the glycine-rich segments of approximately 20-30 amino acids. Orange boxes represent the poly-alanine blocks with 4-15 consecutive alanine residues. The picture is not drawn to scale and the proteins comprise approximately 100-150 tandem repeats of poly-Ala/Gly-rich motifs, according to Ayoub *et al.* (2007).

Major ampullate spidroins consist of non-repetitive amino- (N-) and carboxy- (C-) terminal domains, considered to have distinct tertiary structures (Rising et al., 2006, Sponner et al., 2005c, Ittah et al., 2007) and a large, highly repetitive and amphiphilic region dominated by amino acids with small or no side-chains (*i.e.* Ala and Gly) (Andersen, 1970, Lombardi

and Kaplan, 1990, Casem et al., 1999), located in between the terminal domains.

The MaSps are large, and by using different experimental procedures, their size have been estimated to 260-720 kDa (Candelas et al., 1983, Mello, 1994, Jackson, 1995, Sponner et al., 2005a), and by SDS-PAGE under reducing and non-reducing conditions, they display a disulphide-dependent dimeric nature (Sponner et al., 2005a). It should be mentioned that size determinations of high molecular weight proteins by SDS-PAGE are imprecise and proteins rich in alanine and glycine can display abnormal running behaviors (Mello, 1994, Jackson, 1995). This can, in part explain the wide range of molecular weights determined for dragline spidroins.

The first partial gene sequences to be described code for two distinct structural proteins of the *N. clavipes* dragline (Xu and Lewis, 1990, Hinman and Lewis, 1992), and thereafter, sequences encoding fibroin proteins of *A. diadematus* was obtained (Guerette et al., 1996). The sizes of the encoding mRNAs are large and have been estimated to 7.5-12 kb, using Northern blotting (Guerette et al., 1996, Hayashi et al., 1999). Since then, a vast amount of sequences, encoding structural dragline proteins, have been determined. But until recently, only partial dragline protein genomic- and cDNA sequences have been available. This is most likely due to the difficulties associated with cloning long stretches of repetitive DNA, rich in C and G. The majority of the available sequences encode the C-terminal part, as a consequence of technical cloning issues favoring 3'-amplification of mRNA. The high abundance of alanine and glycine in the spidroins is reflected by the usage of the nucleotides G and C in the first two codon positions. However, the third position for these codons are strongly biased towards A or T (Hinman and Lewis, 1992, Ayoub et al., 2007, Rising et al., 2007). As a result, the DNA melting temperature drops and the degree of secondary structure of the mRNA is most likely reduced, thereby facilitating translation (Hinman and Lewis, 1992).

Recently, the first full-length sequences coding for MaSp1 and MaSp2 were isolated from a genomic library derived from the cobweb weaving black widow (*L. hesperus*). The genes were found to consist of one big exon of coding sequence, containing approximately 9400 bp (MaSp1) and 11300 bp (MaSp2) (Ayoub et al., 2007). Distinct repeat units were found in each protein, which further strengthen the general view that separate genes encode each silk protein (Xu and Lewis, 1990, Hinman and Lewis, 1992,

Guerette et al., 1996). The proteins also contained conserved non-repetitive N- and C-terminal domains, which displayed similarity to previously published sequences.

1.3.1 The N-terminal domain

Attempts to sequence the protein constituents of the major ampullate gland and the dragline silk using Edman degradation have not been successful in identifying the N-terminal domain (Mello, 1994, Sponner et al., 2005a), and whether or not the N-terminal domain is part of the solid silk has never been determined. Gene sequences coding for the N-terminal domain of dragline silk proteins have only recently been isolated and codes for some 130 amino acids (Motriuk-Smith et al., 2005, Rising et al., 2006, Ayoub et al., 2007). The N-terminal domain displays a non-repetitive sequence without the typical amino acid-motifs, characteristic for the repetitive region. The N-terminal was predicted to contain predominantly helical secondary structures (Motriuk-Smith et al., 2005, Rising et al., 2006), and from helical wheel analysis and circular dichroism spectroscopy it was proposed to fold into a bundle of five α-helices that are connected by relative short loops (Rising et al., 2006). The discovery of signal peptides in the N-terminal revealed its importance for the proteins entrance to the secretory pathway. This is in agreement with the observation of epithelial cells lining the gland wall with extensive endoplasmic reticulum and large numbers of secretory vesicles. These are morphological characteristics compatible with ongoing synthesis of secretory proteins (Bell and Peakall, 1969, Plazaola and Candelas, 1991). The signal peptide is likely to be removed since appropriate consensus cleavage sites were found (Rising et al., 2006). Obvious sequence conservation was also noted when aligned with previously published N-terminal sequences (Rising et al., 2006), and when the first full-size gene sequences of dragline spidroins were isolated, putative regulatory elements were discovered in non-coding 5'-flanking sequences (Ayoub et al., 2007).

1.3.2 The C-terminal domain

All spider dragline silk proteins seem to share a conserved C-terminal domain, approximately 100 amino acids long. Even though C-terminal sequences of flagelliform silk differ from the other types of spider silk studied, the domains are expected to have similar physical properties and may perform similar functions (Challis et al., 2006). The C-terminal domain

has been detected in glandular protein extracts as well as in the dragline silk and displayed dimers via disulfide bridge formation (Sponner et al., 2004).

A number of possible functions have been assigned to the C-terminal domain and most of them are addressing its importance for protein solubility and in fiber assembly. It has been proposed that the C-terminal domain is important in maintaining the soluble or liquid crystalline state of silks prior to extrusion (Kerkam et al., 1991), and as with the N-terminal domain, the C-terminal domain is suggested to facilitate the formation of multi-molecular complexes in the form of micelles, where the two hydrophilic domains reside in the periphery of the micelles, thereby enclosing the repetitive amphiphilic regions of the protein within the micelle (Jin and Kaplan, 2003, Bini et al., 2004, Sponner et al., 2004, Sponner et al., 2005c). This would enable storage of proteins at high concentrations. Additional functions such as a potential role in signaling have been suggested (Beckwitt and Arcidiacono, 1994) and it has been speculated that the highly conserved hydrophobic regions could be required for recruiting accessory proteins such as chaperonins, in order to facilitate correct protein folding (Challis et al., 2006). However, no experimental evidence for such functions has been presented.

Despite the conservation of the N- and C-terminal domains, no function have been conclusively determined, and the possibility exist that they have multi-functional roles in spider silk processing.

1.3.3 The repetitive region

The repetitive segment of dragline spidroins is dominated by iterations of alanine- and glycine-rich regions. The alanines exist almost exclusively in blocks of 4-15 consecutive residues $(A)_n$ or in a poly-Gly-Ala motif $(GA)_n$ (Xu and Lewis, 1990, Hinman and Lewis, 1992, Guerette et al., 1996, Rising et al., 2007, Gatesy et al., 2001). $(A)_n$- and $(GA)_n$-motifs are postulated to create β-sheets that stack together and thereby form rigid crystals in the fiber (Parkhe et al., 1997, Simmons et al., 1994, Simmons et al., 1996). These crystals link protein molecules together and could be seen as nodes in a molecular network, conferring the silk its strength (O'Brien et al., 1998).

The glycine-rich stretches are some 20-30 amino acids long and can be further subdivided into two general motifs, GGX and GPGXX (X refers to

a limited subset of amino acids), which are believed to adopt specific structures that have a profound impact on the properties of the fiber (Hayashi et al., 1999, Kümmerlen et al., 1996, van Beek et al., 2002). The successive GPGXX-motif is postulated to create β-turn spirals stabilized by internal hydrogen bonding. The consecutive turn structures are suggested to provide an elasticity module to the silk similar to the β-turn spiral of elastin (Hayashi et al., 1999). The number of successive GPGXX-repeats have been found to correspond with elasticity for flagelliform silk, which is extremely extensible (200-500%) (Vollrath and Edmonds, 1989, Köhler and Vollrath, 1995, Gosline et al., 1999) and dragline silk that stretches about 35% before breakage. Flagelliform silk could have approximately 4-5 times longer spring-like spirals, composed of consecutive β-turns, than found in dragline silk (Hayashi et al., 1999). Although the results from biophysical studies of the glycine-rich regions not always are consistent, the existence of turn structures in the glycine rich regions is supported by NMR spectroscopy data (Michal and Jelinski, 1998).

The GGX-motif is suggested to form 3_1-helical structures that could interact to maintain alignment among adjacent protein chains in the fiber (Kümmerlen et al., 1996, Hayashi et al., 1999). An interesting suggestion is that flagelliform silk, which contain GPGXX- and GGX motifs, but not the β-sheet forming $(A)_n$ or $(GA)_n$ motifs (Hayashi et al., 1999), could be stabilized through the interactions between adjacent GGX-stretches in neighboring protein chains (Lewis, 2006). Moreover, besides conferring stabilizing intermolecular hydrogen bonds in the "amorphous" region (see further below), the GGX-motif has been suggested to also be included in β-sheet regions (van Beek et al., 2002).

Whereas $(A)_n$, GA and GGX are found in MaSp1, $(A)_n$ and GPGXX are the repeats typically found in MaSp2. It has been proposed that the proline-containing motif is a determinant for MaSp2 (Hayashi and Lewis, 1998, Gatesy et al., 2001, Bini et al., 2004). The four distinct motifs found in dragline silk have probably been conserved through stabilizing selection during at least 125 million years and are therefore likely to be critical to the mechanical properties of the fiber (Gatesy et al., 2001). The importance of the different motifs found in the repetitive region and their implications on fiber structure and function will be further addressed below.

1.4 Protein storage

Silk proteins, synthesized in the columnar epithelial cells in the tail and sac of the gland, are assembled in secretory granules that are subsequently discharged into the lumen (Plazaola and Candelas, 1991, Bell and Peakall, 1969). The protein feedstock is transported to, and stored in the sac at concentrations of approximately 30-50% (w/v) (Chen et al., 2002b, Hijirida et al., 1996) until fiber formation is initiated. The spider silk protein feedstock is considered to become a lyotropic, liquid crystalline protein dope (Kerkam et al., 1991, Vollrath and Knight, 2001) after leaving the gland. It means that it forms a substance that flows as a liquid but maintains some of the orientational order characteristic of a crystal, with the long axes of adjacent molecules aligned approximately parallel to one another. This would allow the molecules to align while the protein dope slowly flows through the storage sac and down the duct (Knight and Vollrath, 1999, Vollrath and Knight, 2001).

How spiders manages to store these proteins, highly susceptible to aggregation and with the main purpose to polymerize into a solid fiber, at these high concentrations is not fully resolved. Theories suggest that the proteins associate into multi-molecular complexes in the lumen of the gland in order to bury the hydrophobic regions of the amphiphilic proteins and expose the hydrophilic parts to the aqueous surrounding, thereby preventing aggregation that would be detrimental for the spider (Knight and Vollrath, 2002, Jin and Kaplan, 2003, Bini et al., 2004, Foo et al., 2006). It has been suggested that the silk proteins of orb-web spiders, when stored in the gland, assume rod-like shapes and assemble together into liquid crystalline units in the form of bi-layer discs. As fiber formation initiates environmental conditions change, and together with an increase in elongational flow and shear forces along the duct, the rod-shaped spidroin molecules get mechanically denatured and increasingly aligned. Intra- and intermolecular connections increase, followed by a phase transition and solidification of the fiber (Knight and Vollrath, 2002).

In a study where silk fibroins from *B. mori* had been dissolved in lithium bromide, dialyzed, and blended with polyethylene oxide (PEO) in different concentrations, globules with a micellar substructure could be observed with scanning electron microscopy (SEM) and atomic force microscopy (AFM). These globular structures increased in size with decreasing PEO concentration. Micellar morphology was also seen in silk glands after dissection (Jin and Kaplan, 2003). It was suggested that, from having a more

extended chain structure, the hydrophobic regions begin to assemble and the hydrophilic terminal domains are located to the outer edges of the micelles. The small hydrophilic stretches, interspersed among the hydrophobic regions, are suggested to entrain water into the micelles and thereby enhance the solubility and prevent premature aggregation. Dehydration and subsequent increase in protein concentration force the micelles to aggregate into larger globules, and further physical shear together with possible environmental changes, leads to the formation of fibers (Jin and Kaplan, 2003, Bini et al., 2004, Foo et al., 2006). Since the general architecture of fibroins from the *B. mori* silkworm and the dragline silk spidroins of the spider is similar, it was suggested that the assembly of multi-molecular complexes could occur in a similar fashion in both organisms (Jin and Kaplan, 2003, Bini et al., 2004).

A stabilizing role of the spidroins predominantly disordered state by divalent cations (*i.e.* Mg^{2+} and Ca^{2+}) has been suggested (Dicko et al., 2004). Stabilizing effects by Ca^{2+} have also recently been observed for dissolved fibroin from the *B. mori* silk worm, and the divalent nature of the cation was proposed to induce intermolecular ion-bridge cross-linking (Matsumoto et al., 2008). Cross-linking may reduce the mobility of the protein chains and intermolecular accessibility, preventing molecular self-assembly and β-sheet crystallization by decreasing shear sensitivity (Matsumoto et al., 2008).

In summary, storage of protein feedstock and formation of silk fibers is believed to rely on multi-molecular structures such as micelles and/or molecular rods that are dependent on the packing and conformation of the individual protein units. The relationship, however, between the shapes of these superstructures and the various forms of protein conformation remains elusive.

1.5 Dragline formation

Fiber formation is not only dependent on the chemical composition of the silk constituents (*i.e.* primary structure of the proteins). In order to establish a bio-mimetic production system we must also understand the fundamental functions and conditions under which the soluble bulk silk secretions are irreversibly converted into a solid fiber. Spiders are capable of turning a liquid crystalline protein dope into a fiber with remarkable mechanical

properties under physiological conditions without the use of harsh solvents under ambient pressure and temperature (Kerkam et al., 1991).

Wilson (1969) suggested that spinning of the dragline depends on three factors: (i) the body pressure of the spider forcing liquid silk up the duct from the gland; (ii) the control-valve regulating the flow of liquid silk; and (iii) the tension in the silk thread aligning the protein molecules. This general view has later been supplemented by studies indicating that the fiber formation process is more intricate and depend on multiple factors. Features such as extensional flow and shear forces, water extraction, protein concentration, reduction in pH and altered ion concentrations are considered key driving forces in the process of *in vivo* fiber formation.

Perhaps the foremost factors in creating a solid fiber is the extensional flow and shear forces occurring in the duct, aligning the molecules. The progressive increase in birefringence of the liquid crystalline feedstock when converted to a solid fiber would indicate increased molecular alignment, which has been suggested to occur in the duct during fiber formation (Work, 1977b). Later studies have strengthened this theory where it is suggested, based on Congo Red staining of the silk protein along the fiber production pathway, together with force measurements, that the main conversion from liquid crystalline silk to solid fiber occurs in the duct where a strain-induced phase separation takes place (Knight et al., 2000). Furthermore, it is suggested that the geometry of the progressively narrowing duct maintains a constant and slow elongational flow rate that would prevent premature crystallization of the silk proteins into β-sheets before the start of the draw down taper, which due to its rapid narrowing would induce the β-transition (Knight and Vollrath, 1999, Knight et al., 2000).

Based on studies of anatomy and morphology of the major ampullate gland, the duct has been suggested to be involved in water removal from the protein feedstock (Bell and Peakall, 1969, Casem et al., 2002, Vollrath et al., 1998). In addition to reduction of water content in liquid silk, a change in ion-composition during the solidification of the fiber has been observed. The presence of an ATP driven pump was suggested to be responsible for the observed increase in $[K^+]$ and decrease in $[Na^+]$ during fiber formation, but no function of the ion exchange was postulated (Tillinghast et al., 1984). These findings have been supported by later studies on the fiber formation in *N. edulis*, where it was found that an increase in K^+ and phosphate

concentrations was associated with a decrease in Na^+- and Cl^- concentrations. The uptake of Na^+ with a parallel secretion of the more chaotropic K^+ to the lumen of the duct could lead to a conversion of structured water on the surface of the proteins into bulk water (Knight and Vollrath, 2001, Foo et al., 2006). This effect could further be reinforced by an observed decrease in pH from 6.9 in the ampulla of the gland to 6.3 for the luminal content of the third limb of the duct (Knight and Vollrath, 2001). The observed decrease in pH during the formation of the dragline could be attributed to proton pumps located in the final limb of the duct, as previously demonstrated in *A. diadematus* (Vollrath et al., 1998). The increased $[K^+]$ with concomitant decrease in pH, coupled with the high strain rate in the internal draw down taper (Knight and Vollrath, 1999) would favor the unfolding and β-crystallization of the spidroin molecules in the spinning dope, leading to the formation of the solid fiber (Knight and Vollrath, 2001, Vollrath and Knight, 2001, Chen et al., 2002b).

Some arthropods do rely on cross-linking of molecules in resilient structural materials through Tyr-Tyr interactions, possibly enhancing the mechanical properties of the polymers (Andersen, 1964, Andersen, 2004, Lassandro et al., 1994). Together with indication of peroxidase activity in the major ampullate gland of the *N. edulis* spider (Vollrath and Knight, 1999) and the discovery of a cDNA coding for a peroxidase expressed in major- and minor ampullate glands of *N. senegalensis* (Pouchkina et al., 2003), it was suggested that spiders might interconnect spidroins covalently through Tyr-Tyr interactions. Observations suggesting that Tyr residues are accessible further supported this hypothesis (Dicko et al., 2004). However, evidence for such interactions have not been found (Xu and Lewis, 1990, Vollrath and Knight, 1999).

1.6 Dragline silk and structure-function relationships

A large number of biophysical studies have been conducted in order to elucidate the protein structure in soluble form and in the fiber. In spite of that, the secondary structures of the proteins in the dope or in the fiber have not been indisputably determined. However, the consensus view is that the proteins in the gland are in a random and/or helical conformation, whereas the proteins in the fiber have a more ordered structure where β-sheets, turn structures and 3_1-helices predominate.

From a combinatory study, using ^{13}C-NMR, Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopy, it was suggested that the silk dope in the gland exist in dynamically averaged helical conformations. No evidence for β-sheet structures in the soluble proteins of the ampullate gland was found (Hijirida et al., 1996). Further NMR studies on the major ampullate gland silk from *L. hesperus* (Lawrence et al., 2004) and *N. edulis* (Hronska et al., 2004) propose a predominantly random coil, dynamically disordered conformation for the silk solution in the gland. CD analyses have proposed mainly random coil and α-helical conformations of the proteins in the anterior part of the gland, whereas dramatic structural changes of the spidroins occur in the silk production pathway, resulting in the formation of β-sheet rich structures found in the glands posterior part (Kenney et al., 2000, Kenney et al., 2002). Raman confocal spectroscopy and vibrational CD studies have been used to study the conformation of the proteins *in situ* in the intact major ampullate gland of *N. clavipes* and *A. diadematus*, together with recombinant variants of MaSp1 and MaSp2. It was concluded that the proteins are in a natively unfolded state with a conformation composed of random- and polyproline II segments with some α-helices (Lefevre et al., 2007a), and additional Raman spectroscopy studies, further strengthened this hypothesis (Lefevre et al., 2008).

One contributing fact to the difficulties of assigning any specific conformations to soluble silk proteins could be the difficulties in handling these proteins during experimental work without causing any structural changes due to shear forces or dehydration (Chen et al., 2002b, Lefevre et al., 2007a, Lefevre et al., 2008).

In the solid dragline, morphological features such as elongated cavities called canaliculie orientated parallel to the silk fiber axis (Shao et al., 1999a, Sponner et al., 2007) and skin-core structures (Frische et al., 1998, Li et al., 1994, Sponner et al., 2007, Work, 1984) have occasionally been observed. Canaliculie are thought to be formed when the spherical droplets in the liquid crystalline dope within the ampulla are progressively stretched by elongational flow in the duct during fiber formation (Knight and Vollrath, 1999, Vollrath and Knight, 1999). It has been suggested that canaliculie could inhibit the spreading of cracks and delay rupture of the thread during load (Shao et al., 1999a).

By examining dragline silk with light microscopy and SEM after repeated wetting and stretching of the fiber, a skin-core structure could be deduced

(Work, 1984). Additional studies employing biophysical methods such as, AFM, TEM, SEM and NMR spectroscopy also indicate hierarchical structure arrangements of dragline silk, involving fibrillar core and occasional skin structures (Li et al., 1994, van Beek et al., 2002, Augsten et al., 2000), and by labeling with concanavalin A lectin-gold complex, the presence of glycoproteins could be confirmed in the skin and inside the fiber (Augsten et al., 2000). Other intricate four-layer models of the dragline silk's structural organization have been suggested (Vollrath et al., 1996, Vollrath and Knight, 1999). A three-layer morphology of the dragline silk was proposed by Sponner and co-workers, when analyzing cross-sections of the dragline with specific antibodies for MaSp1 and MaSp2, respectively, visualized by electron microscopy with gold-labeled secondary antibodies. An outer glycoprotein coat with an inner protein skin-layer surrounding the core-region was suggested, since immunostaining with spidroin specific antibodies was not generally observed in these two outer layers (Sponner et al., 2005b). Resent studies have extended these previous theories to a five-layer dragline morphology (Sponner et al., 2007). Frische *et al.* (1998) was unable to detect a fibrillar structure, but did find a thin outer layer of higher electron density consistent with a skin-core model. Studies on dragline silk employing different spectroscopic and microscopic techniques did not detect a skin-core structure nor fibrillar substructures (Thiel et al., 1994), and when analyzed by X-ray diffraction, electron microscopy and molecular modeling, the lack of skin-core structures in the dragline was further supported (Thiel et al., 1997).

It should be kept in mind that due to imperfections in sectioning techniques, morphological artifacts could arise in the material and wrongly interpreted as true morphological structures. Therefore, the difficulties in assessing morphological structures with these methods should be appreciated.

MaSp1 and MaSp2 are uniformly distributed in the gland and duct when still in their soluble form, with MaSp1 seemingly more abundant (Sponner et al., 2005a, Brooks et al., 2005). In the solid fiber however, MaSp2 is found in clusters concentrated to the core centre and MaSp1 still seems homogenously dispersed throughout the entire core (Sponner et al., 2005b, Sponner et al., 2007). It is not known how the proteins separate into distinct areas in the fiber, but it is suggested that the segregation of the two proteins could have implications on the mechanical properties of the fiber. The proposed capability to regulate the crystalline state of the silk could

affect the rigidity of the fiber and allow for movement of the stiffer parts by introducing an elastic element (Sponner et al., 2005b, Sponner et al., 2007). In addition, similar to the proposed function of canaliculie (Shao et al., 1999a), it is suggested that MaSp2 might introduce heterogeneities that could help avoid lateral crack propagation in the dragline (Sponner et al., 2005b).

In the middle of the 20th century attempts at deducing the secondary structure of the proteins in the solid silk were made. Based on initial X-ray diffraction studies (Warwicker, 1960, Lucas, 1964) it was concluded that silk fibers have a general composite structure consisting of crystalline- and non-crystalline structures where the crystalline region arise through stacking of pleated β-sheets mainly composed of small side-chain amino acids. The observed crystals of stacked β-sheets could be viewed as rigid nodes in an amorphous network linking protein chains together creating a semi-crystalline biopolymer (Gosline et al., 1984, O'Brien et al., 1998). This general structure has been confirmed by later diffraction studies (Bram et al., 1997). Parkhe *et al.* (1997) noted that in the dry fiber the β-sheet crystals are oriented parallel to the fiber axis. Solid-state NMR data further suggested that the poly-Ala stretches observed in the protein sequence were responsible for the formation of these β-sheets.

Studies of spider dragline silk employing FTIR (Dong et al., 1991), NMR (Simmons et al., 1994, Simmons et al., 1996, Kümmerlen et al., 1996, Lawrence et al., 2004, Bonev et al., 2006) and Raman spectroscopy (Shao et al., 1999b, Lefevre et al., 2007b) clearly indicate that spider dragline silk comprises crystallites formed by stacked β-sheets composed of alanine-rich protein stretches. It has been suggested that glycine repeats adjacent to the poly-Ala blocks could also be incorporated into the crystalline regions, as judged by TEM (Thiel et al., 1994, Thiel and Viney, 1996, Thiel et al., 1997), solid-state NMR (Simmons et al., 1996, van Beek et al., 2002, Holland et al., 2008a) and Raman spectroscopy (Lefevre et al., 2007b).

The over-all content of β-sheets is higher than the degree of crystallinity, suggesting that some of the β-sheets are located in the amorphous regions (Lefevre et al., 2007b). This is in accordance with previous findings that alanine-rich β-sheets are found in two distinct crystalline populations. Solid-state ^{2}H-NMR spectroscopy showed the existence of a poorly oriented crystalline phase, denoted protocrystals, comprising 60% of the alanine residues in unaggregated β-sheets, and a highly oriented β-sheet population

incorporating 40% of the alanines into rigid crystals, aligned parallel to the fiber axis (Simmons et al., 1996).

Previously, due to the inability of available biophysical methods to assess distinct structures other than the rigid crystallites, the remainder of the silk structure was depicted as unstructured or "amorphous". However, theories regarding the structure of the "amorphous" matrix have recently emerged, suggesting it to be more ordered and organized than previously stated. Van Beek *et al.* (2002) suggest that all domains present in dragline silk have a preferred secondary structure and are strongly oriented, with the chains predominantly parallel to the fiber. These experiments have provided evidence that a fraction of glycine is present in elongated helical conformations, in agreement with previous findings (Kümmerlen et al., 1996). This conformation were suggested to be stabilized by inter-chain hydrogen bonding, which in turn would allow for efficient packing. The defined 3_1-helical secondary structure was found to be ordered and also rigid (van Beek et al., 2002). Resent studies also found 3_1-helical structures assigned to the prevalent GGA-motif, however in a disordered state (Holland et al., 2008a). Data also suggests that motifs in the glycine-rich regions could form turn-like structures (Michal and Jelinski, 1998). This would support the assumed presence of β-turn spirals suggested to originate from the GPGXX motif found in MaSp2 and in flagelliform silk (Hinman and Lewis, 1992, Hayashi and Lewis, 1998, Hayashi et al., 1999). Raman spectroscopy studies investigating dragline silk agree with the notion that the protein backbone in the "amorphous" regions exhibits a significant level of orientation, although less than in the β-sheets (Lefevre et al., 2007b).

To summarize, the combination of high tensile strength and extensibility is what gives the dragline its unique mechanical properties. This dual nature arises due to the silks composite architecture where two structurally distinct regions can be discerned. The "amorphous" region, suggested to confer extensibility to the silk, is composed of glycine rich stretches with proposed amino acid motifs such as GPGXX and GGX, where the former have been suggested to form sprig-like β-turn structures and the latter 3_1-helices (Hayashi et al., 1999, van Beek et al., 2002). In the "amorphous" region, rigid crystallites are dispersed, suggested to be composed of stacked β-sheets formed by poly-Ala and/or poly-Gly/Ala segments. The crystallites link protein chains together (Figure 4) and is thought to give the dragline its strength (Simmons et al., 1996, O'Brien et al., 1998).

1.6.1 Supercontraction

Supercontraction, first recognized by Work and co-workers (Work, 1977a, Work, 1981), affects the morphology and mechanical properties of dragline silk when subjected to water (Figure 4). The contraction leads to a radical reduction in stiffness and a large increase in extensibility (Gosline et al., 1984).

When designing synthetic protein polymers for various applications with specific mechanical properties, inspired by sequences from the structural proteins of the spider dragline, supercontraction remains an impediment. An understanding of how differences in the primary structure of these proteins impact on supercontraction is therefore critical (Yang et al., 2000).

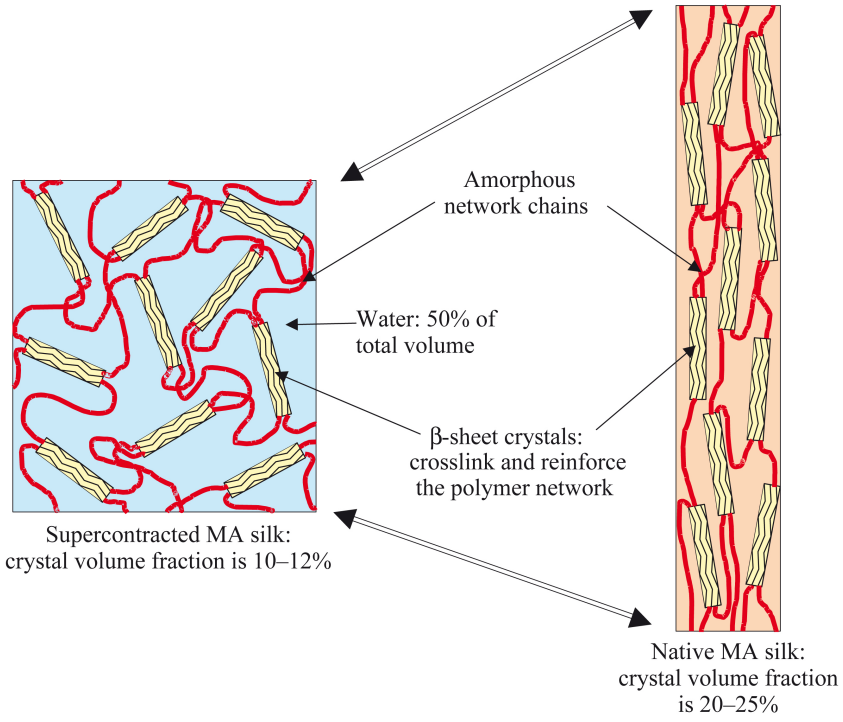

Figure 4. The effect of supercontraction on *A. diadematus* dragline silk integrity and structure when hydrated. In the dry silk, the rigid β-sheet crystals are aligned parallel to the fiber axis (right hand side of picture) and when hydrated the crystals loses their orientation as stabilizing hydrogen bonds in the glycine-rich regions are broken (left hand side of picture). Adapted from Gosline *et al.* 1999.

The interaction of dragline with water leads to a swelling that doubles the fiber volume with a concomitant length decrease of about 50% (Work and Morosoff, 1982). This is a reversible process that upon drying, stretched to its original length, leads to an almost total recovery of the fiber properties (Work, 1981, Work and Morosoff, 1982). X-ray diffraction and birefringence studies showed that the molecular organization is reduced when the fiber is wetted (Work and Morosoff, 1982, Parkhe et al., 1997, Grubb and Ji, 1999, Fornes et al., 1983). The observed crystalline domains, aligned parallel to the fiber axis in the dry dragline, are largely unaffected by hydration and merely rotate when the fiber is wetted. No change in the β-sheet spacing was observed implying that the β-sheet regions are inaccessible to water. If the fiber was stretched back to its original length and dried, the orientation of the β-sheet was restored (Parkhe et al., 1997).

It was assumed that the "amorphous" region must be appreciably oriented in the dry fiber and that, in the formation of the fiber, the molecules are effectively locked into an oriented state by intermolecular bonds. Hydrogen bonds are suggested to form when the fiber dries in a stressed state, and if wetted, the water molecules act as a plasticizer, breaking the hydrogen bonds and allowing the system to become more randomly oriented (Fornes et al., 1983). The results are consistent with Raman spectroscopy (Shao et al., 1999b) and NMR data (Parkhe et al., 1997, Eles and Michal, 2004, Holland et al., 2008b). Minor ampullate silk is plasticized by water in a similar manner as major ampullate silk (dragline), and reorientation of its sheet regions when hydrated have also been observed (Parkhe et al., 1997, Holland et al., 2008b). However, contrary to dragline silk, minor ampullate silk does not supercontract (Work, 1977a). This indicates that although the penetration of water into the Gly-rich region of the silk could help explain the supercontraction process, this observation alone cannot account for supercontraction in dragline (Holland et al., 2008b).

It has been observed that dragline silk from different spider species, with differing mechanical properties, displayed significant differences in behavior in the native state as well as during and after supercontraction (Shao and Vollrath, 1999). It was suggested that the silk, which displayed the lowest degree of supercontraction should contain the highest amount of rigid β-sheet regions. This assumption was supported when native and supercontracted dragline silk from four different spiders and the filament of

B. mori were analyzed with Raman spectroscopy (Shao et al., 1999b). It is speculated that hydration-induced contraction of dragline silk has ecological functions such as taking up slack in the web and restore its tension after prey capture or precipitation (Lewis, 1992, Gosline et al., 1999), but it could also be merely a trade of consequence of evolving a high performance composite polymer which is both strong and extendible (Gosline et al., 1999, Shao and Vollrath, 1999, Liu et al., 2005). Studies have shown that deformations in the dry silk can be reversed by water-induced supercontraction, leading to a recovery of the tensile properties (Elices et al., 2004).

1.6.2 Proline

In two recent studies (Savage and Gosline, 2008a, b) it is indicated that there are major differences in the structural organization of the Gly-rich network chains and the mechanism of elasticity in proline-rich and proline-deficient draglines from different spider species. *A. diadematus* dragline silk contains about 16% proline, whereas *N. clavipes* contains 3.5%. Dry *N. clavipes* had roughly 50% higher birefringence than dry *A. diadematus* dragline, and difference in birefringence increased even more upon hydration. Since both silks have similar amounts of poly-Ala, β-sheet crystals, it was assumed that the differences are in the Gly-rich network structure of these two silks. Although the mechanical properties of these two silks in dry state were found to be indistinguishable, large differences between the hydrated silks were observed. It was suggested that a difference in the stability of the hydrogen bonds exists in the network chains of these two silks in their dry state. *A. diadematus* dragline was proposed to contain essentially amorphous random coil network chains that become highly mobile when hydrated and exhibit rubber-like elasticity as described by Gosline *et al.* (1984). Proline residues present in the Gly-rich network chains of spider silk is thought to destabilize secondary structures and favor a more amorphous network structure (Savage and Gosline, 2008a). Accordingly, *N. clavipes* dragline displayed quite stiff and ordered network chains in both dry and hydrated state, which is likely due to the low proline content allowing stable structures to form. In accordance, when probing the same subject (Liu et al., 2008a, b), it was suggested that proline, as part of the Gly-rich region, prevents tight packing and facilitate water access to the Gly-rich region in which hydrogen bonds are broken, which in turn results in an concomitant softening and contraction of the silk.

1.7 Mechanical testing of spider silk properties

The vast research interest in spider silk, and in particular for the dragline, sprung from the observations of its remarkable and unique mechanical properties (Denny, 1980). However, assessing the mechanical properties of spider silks has proven cumbersome, since a lot of factors seem to influence fiber performance (Madsen et al., 1999, Madsen and Vollrath, 2000). Differences in mechanical properties of draglines from different species may be attributed to variations in primary structure. However, variation in mechanical properties in dragline silks from the same species, as well as in different draglines from the same individual has been observed (Madsen et al., 1999). A number of factors affecting the properties and composition of dragline silk have been suggested, such as reeling speed, starvation and diet-variation, forced silking or natural spinning, anesthesia of the spider, temperature, humidity, UV-radiation and silk age. Fiber defects, inaccurate diameter determinations and strain-rate during tensile testing are factors that also affect the results of mechanically testing spider silk properties (Madsen et al., 1999, Cunniff et al., 1994, Madsen and Vollrath, 2000, Craig et al., 2000, Gosline et al., 1999, Vollrath et al., 2001, Pérez-Rigueiro et al., 2006, Pérez-Rigueiro et al., 2007, Agnarsson et al., 2008). Contaminations of the dragline, during forced silking, by silks from adjacent spigots is also suggested to be a complicating factor that could lead to inaccurate interpretations of obtained results (Craig et al., 2000).

When testing spider silks mechanical properties, a lot of information can be gained by conducting stress-strain measurements. The stress (σ) is defined as the force (F) divided by the cross-sectional area (A) of the fiber ($\sigma = F/A$). Normally, the cross-sectional area is considered to be constant throughout tensile testing, even though A will decrease somewhat during extension. The strain (ε) is the deformation, defined as the ratio of change in length (ΔL) to the initial length (L_0), $\varepsilon = \Delta L / L_0$. Initial modulus (E), which can be obtained from the slope of the stress-strain curve in the region where the fiber is considered to be elastic, is a measure of the stiffness of the fiber. When testing spider silks, the first yielding point, often observed in stress-strain profiles as the beginning of a distinct slope change, indicates the transition of the material from being elastic to become irreversibly deformed if increased force is applied. The area under the stress-strain, curve up to the point of breakage, is a measure of the materials toughness and indicates the energy absorbed by the fiber before it fails (Figure 5).

The way energy is absorbed by the spider silks when a flying insect is caught in the web is an important consideration. The kinetic energy could either be dissipated as heat through friction or it could be stored through elastic deformation. Hysteresis is defined as the ratio of energy dissipated to the energy absorbed. It has been shown that the dragline silk of certain spiders transform about 65% of the kinetic energy to heat, and is therefore not available to throw the pray out of the web through elastic recoil. The high levels of hysteresis observed for dragline silks is thought to arise from internal molecular friction (Denny, 1980, Gosline et al., 1999).

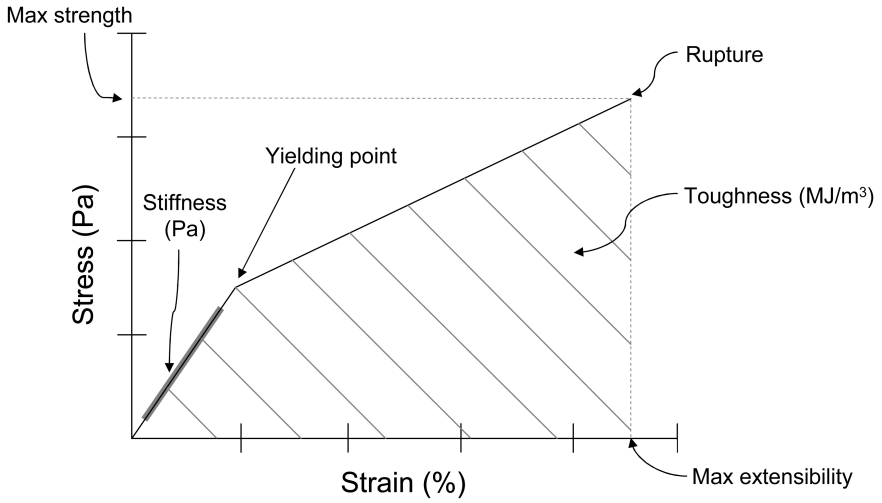

Figure 5. A schematic stress-strain curve of typical appearance when mechanically testing dragline silk. The maximum strength (σ_{max}) is stress at rupture. The maximum extensibility (ε_{max}) is the strain at rupture. Stiffness, or initial modulus (E), is the slope of the stress-strain curve over the first linear portion before the yielding point that denotes the beginning of inelastic deformation. Toughness is the area under the stress-strain curve and indicates the energy required to break the fiber.

An alternative way of assessing fiber mechanical properties is based on the entity denier, which is defined as the weight in grams of a given fiber that is 9 kilometers long. The silk of *B. mori* has a value of 1 denier and a human hair approximately 40-50. *A. diadematus* dragline silk was found to have a denier of 0.07, and if it was long enough to reach around the world at the equator it would only weigh approximately 340 grams (Lucas, 1964). The mechanical properties of dragline silk together with other biological and man-made materials are listed in Table 2.

Table 2. *Mechanical properties of various materials*

Material	Strength σ_{max} (GPa)	Stiffness E (GPa)	Extensibility ε_{max} (%)	Toughness (MJ/m^3)	Ref.
Dragline silk[1]	0.8-1.5	7-11	22-39	140-284	a, b, d, e, f, g, i
B. mori silk	0.6	7	18	70	a
Mammalian tendon	0.12	1.2	13	6	c
Synthetic rubber	0.05	0.001	850	100	a
Kevlar	3.6	130	2.7	50	a
High-tensile steel	1.5	200	0.8	6	a

[1] Only dragline silk from web constructing spiders are included

[a] Gosline *et al.* (1999)
[b] Agnarsson *et al.* (2008)
[c] Gosline *et al.* (2002)
[d] Lawrence *et al.* (2004)
[e] Swanson *et al.* (2006)
[f] Madsen *et al.* (2000)
[g] Madsen *et al.* (1999)
[h] Savage *et al.* (2008a)
[i] Liu *et al.* (2008b)

1.8 Recombinant production of dragline silk proteins

The remarkable properties of spider dragline silk will find many applications if the material can be produced economically (Fahnestock et al., 2000) and much of applied research has focused on mass-producing silk fibers for industrial use (O'Brien et al., 1998). However, unlike domesticated silkworm caterpillars, spiders cannot be readily farmed at high densities because of their territorial and cannibalistic nature. Moreover, one silkworm cocoon yields 300-1200 m of fiber (Winkler and Kaplan, 2000), which is much more than can be forcibly reeled from a single spider's major ampullate gland. Therefore, to obtain large quantities of spider silk for industrial applications, researchers have been attempting to clone silk genes for over-expression in bacteria, yeast, plants, mammalian cell culture systems and transgenic animals (Hu et al., 2006). Recombinant expression of spidroin analogs most often involve the use of cDNA or synthetic polynucleotides based on consensus repeats from native sequences (Fahnestock, 1994, Lewis et al., 1996, Lazaris et al., 2002, Teulé et al., 2007, Brooks et al., 2008, Bogush et al., 2008).

Recombinant expression of spider silk is cumbersome due to the highly repetitive gene sequences, rich in G and C. The repetitive nature of the genes often leads to genetic instability, with deletions or rearrangements as a result. Furthermore, the GC-rich and repetitive sequences in mRNA chains can induce undesirable secondary structures that hinder translation. The high content of G and C in the genes translates into high levels of alanine and glycine in the spidroins, and to cope with extensive usage of these amino acids, spiders seem to have developed gland-specific pools of tRNA for these residues (Candelas, 1990). In recombinant expression systems however, this uniform amino acid usage puts a lot of strain on the translation system, which might be depleted of corresponding tRNAs. These limitations can cause the molecular weight distribution of spider silk proteins synthesized in recombinant expression systems to be heterogeneous, with a significant amount of prematurely terminated chains (Arcidiacono et al., 1998, Fahnestock and Bedzyk, 1997, Fahnestock and Irwin, 1997, Fahnestock et al., 2000).

However, advantages can also be drawn from the modular architecture of the spidroin genes. The highly repetitive amino acid sequences allow synthetic genes for these proteins to be constructed rather easily from synthetic oligonucleotides that are then multimerised to form larger genes (Lewis et al., 1996, Fahnestock and Irwin, 1997, Bini et al., 2004). The size of the proteins expressed can thereby without difficulty be varied. Moreover, synthetic genes can be designed with codons preferred by the intended expression host (Lewis et al., 1996) in order to maximize expression levels (Prince et al., 1995, Fahnestock and Irwin, 1997, Lewis et al., 1996).

1.8.1 Production in bacteria

Expressing partial native (Arcidiacono et al., 1998) or synthetic (Fahnestock, 1994, Fahnestock and Irwin, 1997, Lewis et al., 1996, Prince et al., 1995) gene sequences coding for amino acid sequences based on the consensus repeats in the dragline silk proteins of *N. clavipes* in bacteria, begun the era of biotechnological production of spider silk proteins. Silk protein analogs up to 163 kDa have been produced in *E. coli*, but as gene sizes increases, a general decrease in protein yield is observed.

Synthetic genes have been constructed by using consensus sequences from the repetitive domain of the MaSp1 and -2 proteins produced by *N.*

clavipes. Through multimerisation, genes of varying size could be designed and subsequently expressed in *E. coli*. As the gene-size increased, a general decrease in expression was observed (Prince et al., 1995). Different proteins of approximately 15-41 kDa were expressed to a limited extent (2-15 mg/L culture) even though produced in high cell density fermentations. The low production might be attributed the high GC-content, leading to stable mRNA secondary structures, thereby hampering protein synthesis. The proteins also displayed poor solubility (Prince et al., 1995).

In a similar fashion, a synthetic MaSp2 gene was constructed and resulted in a 58 kDa protein upon bacterial expression. The appearance of lower molecular weight bands was observed after delays in the purification process and they were assumed to arise from proteolytic cleavage, rather than from premature synthesis termination, since truncation was exclusively occurring in the N-terminal. Expression levels of larger inserts were low and suggested to be due to low copy numbers of the plasmid (Lewis et al., 1996). In another attempt to produce synthetic MaSp analogs, the yield and homogeneity of proteins produced were limited by premature termination of synthesis, probably as a result of processivity errors in protein synthesis (Fahnestock and Irwin, 1997).

A partial native spidroin gene from *N. clavipes*, including the C-terminal domain, introduced in *E. coli* displayed instability, and premature truncation of protein synthesis was reported. Denaturing agents were used during the purification procedures, however only low (4 mg/L) amounts of the 43 kDa protein could be obtained (Arcidiacono et al., 1998). In order to increase the recovery of expressed proteins, the same group introduced organic acids during the purification procedures, thereby increasing the protein yield to 15 mg/L. Due to the stability of spider silk proteins, acids could be used to lyse *E. coli*.-cells and enrich for recombinant spider silk proteins while precipitating many of the bacterial proteins (Mello et al., 2004).

The observed thermal stability displayed by certain recombinant spidroin analogs has been utilized during initial steps in the purification process. By incubating *E. coli* lysates at 70-80°C for 10-20 min, contaminating bacterial proteins could be denatured and precipitated while the spidroin analogs remained soluble (Huemmerich et al., 2004a, Teulé et al., 2007). Small recombinant variants of the dragline silk proteins from *A. diadematus* (ADF-3 and ADF-4), consisting of repetitive domains based on consensus sequences fused to the native C-terminal domain for the respective proteins

have been successfully produced in bacteria. By using fed-batch fermentation, protein yields could be increased from 10-30 mg/L culture medium to 140-360 mg/L (Huemmerich et al., 2004a). However, as with most recombinantly produced silk spidroin analogs, due to a general problem with low solubility, purification procedures often includes the use of denaturing agents. Moreover, spidroin analogs have frequently been precipitated or lyophilized in order to facilitate handling and storage without protein loss. Therefore, resolubilization of the proteins in harsh solvents is often necessary for subsequent use (Arcidiacono et al., 1998, Fahnestock and Irwin, 1997, Huemmerich et al., 2004a, Mello et al., 2004, Teulé et al., 2007)

1.8.2 Production in yeast

Being a eukaryotic organism, yeast was anticipated to have a transcription and translation system more suited for expression of genes coding for recombinant spider silk proteins. Moreover, the ability to secrete the proteins into the extra-cellular medium offers additional advantages, such as simplifying purification procedures leading to low-cost recovery (Fahnestock et al., 2000). Like when producing spidroin analogs in *E. coli*, the gene sequences were optimized for *P. pastoris* codon preferences. Expression of synthetic multimers, consisting of repeats from MaSp1 of *N. clavipes*, of 3000 codons in length, was observed (Fahnestock and Bedzyk, 1997), even though genes longer than 1600 codons were expressed less efficiently than shorter genes. Truncated synthesis as a result of ribosome termination errors was not observed when spidroin analogs were produced in *P. pastoris*. Inserted genes were stable even though a variety of sizes of the produced proteins were observed, as a result of gene rearrangements at transformation. The solubility of produced proteins decreased with time and proteins in the insoluble fraction could only be solubilized in 6 M guanidine-HCl. No secretion into extra-cellular medium was attempted and protein recovery was from cell lysates that were cleared from contaminating host-specific proteins by denaturation and precipitation at pH 5 and 60°C. The soluble spidroin analogs were subsequently precipitated with ammonium sulfate and the purity was up to 95%, as judged by amino acid analysis. Once precipitated, the proteins could only be redissolved in denaturing solvents, such as guanidine-HCl or HFIP, and could then be dialyzed against mildly acidic buffers. Production of approximately 1g/L dragline protein analog was obtained in shake-flask cultures.

Synthetic proteins, 28-32 kDa in size, with self-assembling motifs inspired by dragline silk proteins have recently been expressed in *P. pastoris* for subsequent secretion to the cell medium (Werten et al., 2008). Fed-batch fermentations were performed and resulted in 1-3 g/L of secreted target protein. Repeated precipitation and resolubilization steps at low pH separated the target protein from contaminants and resulted in recombinant proteins with high purity. However, problems during purification were encountered when recombinant proteins bound to host-specific compounds. In this case, secreted proteins were precipitated from cell media and the recovered pellet was resuspended in 100% formic acid. Upon dilution with water, contaminating proteins were denatured and precipitated by centrifugation. Successive precipitation and resolubilization steps lead to higher purity and the protein was subsequently lyophilized. No size heterogeneity in the protein population was reported (Werten et al., 2008).

1.8.3 Production in cell culture

Bovine mammary epithelial alveolar cells immortalized with large T (MAC-T) and baby hamster kidney (BHK) cells have been used for the expression of dragline protein analogs derived from *N. clavipes* and *A. diadematus* cDNA, coding for partial MaSp1 and -2, and ADF-3, respectively. All genes contained the non-repetitive C-terminal domain, but varied in the length of the repetitive region, which resulted in recombinant proteins of 60-140 kDa being expressed. However, larger proteins were expressed less efficiently, which might be attributed to inefficient transcription, insufficient secretion of larger proteins, low copy numbers of the construct being transfected, or limitations of the cell translational machinery. The most encouraging result was from BHK cells expressing a 60 kDa protein from ADF-3. This protein was continuously produced from stably transfected cells in a hollow-fiber reactor to the level of 25-50 mg/L. The protein was enriched from the culture media by precipitation with ammonium sulfate and redissolved in aqueous buffer. The purity of recombinant spidroin analogs ranged from 80-90%, after final ion exchange chromatography.

Since insects and spiders belong to the same phylum, better results in the production of dragline protein analogs were anticipated by using a baculovirus expression system and the Sf9 insect cell line (Huemmerich et al., 2004b). cDNA sequences of similar sizes from *A. diadematus*, coding for a repetitive region together with the C-terminal domain of ADF-3 and ADF-4, were used to transfect insect cells. Proteins were produced that

displayed markedly differing solubility properties. Whereas ADF-3 almost exclusively was found in the soluble fraction, ADF-4 was typically insoluble. It was shown that ADF-4 aggregated into microscopic fibers inside the cells. The filaments found in the cytosol had diameters ranging from 200 nm to 1μm and were up to 100 μm in length. No mechanical evaluation were possible due to their minute size, however, filaments of recombinant ADF-4 were suggested to display similar chemical stability as native dragline silk (Huemmerich et al., 2004b).

1.8.4 Production in plants

Production of synthetic dragline protein analogs using plants as bioreactors have been reported (Menassa et al., 2004, Scheller et al., 2001, Yang et al., 2005), where spidroin analogs up to 100 kDa in size have been expressed in tobacco, potato and *Aradopsis*. Proteins, based on the repetitive sequence of *N. clavipes* MaSp1, were expressed in various sizes (Scheller et al., 2001). The sequences were designed for retention and accumulation of recombinant proteins in the endoplasmic reticulum (ER) of the plant cells, and it was reported that more than 2% of the plants soluble proteins could consist of spidroins. In contrast to expression in *E. coli* (Fahnestock and Irwin, 1997), the accumulation level of transgenic silk protein did not depend on size and the proteins remained stable in the plant tissue. Soluble extracts were heated at 95°C for 10 min and cleared from denatured host-specific contaminants by centrifugation. Lowering the pH of the supernatant resulted in precipitation of remaining contaminating proteins, which could be removed by centrifugation. Pure spidroin analogs were precipitated with ammonium sulfate and dissolved in buffer containing 6 M guanidine-HCl (Scheller et al., 2001).

Size heterogeneity of a MaSp1 spidroin analog was observed when expressed in tobacco. It was suggested to be caused by protease activity or premature termination of translation due to specific tRNA starvation. The accumulation of spidroin analogs, derived from *N. clavipes* MaSp1 and -2, in tobacco during field trials were low (Menassa et al., 2004).

In an attempt to increase accumulation of *N. clavipes* MaSp1 analogs in plants, the proteins were targeted for special cellular compartments to prevent protein degradation. Most significantly, transgenic plants engineered with the ER targeting mechanism were able to accumulate a protein analog, 64 kDa in size, in their seeds to a level higher than 15% of total soluble

protein, whereas vacuole targeting resulted in cleavage and degradation of spidroin analogs. No efforts in purification of the produced proteins were reported (Yang et al., 2005).

1.8.5 Production in transgenic animals

Advances in biotechnology have enabled the production of recombinant spidroin analogs in transgenic animals such as goats and mice with subsequent secretion of the protein into milk for recovery (Karatzas et al., 1999, Xu et al., 2007). Founder mice were mated with wild-type mice and it was shown that the trans-gene could be transmitted to their progenies. A hybrid spidroin with the size of 55 kDa, composed of consensus sequences from the repetitive part of *N. clavipes* MaSp1 and -2, was expressed. The maximum concentration of produced protein was estimated to 12 mg/L. Heterogeneous sizes in the protein population were occasionally observed and suggested to be caused by processing errors during protein synthesis (Xu et al., 2007). Compared to alternative expression systems, development time and production costs are likely to be prohibitive for transgenic animals to be suited for large-scale production of spider silk analogs (Fahnestock et al., 2000).

1.9 Producing artificial spider silk

To elucidate the importance of the spinning procedure and to possibly achieve a material with superior mechanical properties, spider dragline silk and recombinant dragline spidroin analogs have been subjected to artificial spinning.

Solid silks are very stable and resilient to chemicals (Mello, 1994, Huemmerich et al., 2004b, Winkler and Kaplan, 2000). To be able to spin artificial silk, native fibers or lyophilized recombinant silk proteins often have to be dissolved in harsh solvents, such as HFIP (Seidel et al., 1998), formic acid (Lewis et al., 1996) or in high concentration salt solutions such as lithium bromide or lithium thiocyanate (Winkler and Kaplan, 2000). In attempts to artificially replicate the native fibers properties, it is often preferred to spin fibers from solutions with high protein concentrations, necessitating noxious solvents (Bogush et al., 2008). Natural fiber formation during the spiders spinning process is partly dependent on dehydration and protein alignment (Vollrath and Knight, 2001). This is recognized in the

attempts to construct artificial spinning apparatus that, in these respects, mimic the spiders spinning mechanism and produces synthetic silk fibers (Liivak et al., 1998). The procedure is often referred to as wet-spinning, and by expelling a concentrated protein solution through a small needle or similar apparatus, the elongational flow and shear forces created by the spiders progressively narrowing duct are duplicated. The emerging fiber is often allowed to enter a bath with a dehydrating coagulant, such as methanol, ethanol or acetone (Lazaris et al., 2002, Seidel et al., 1998, Bogush et al., 2008), to reproduce the natural water removal. The produced fiber is often further subjected to post-spinning manipulations. Conventional manufacture of synthetic polymer fibers depends on one or more post-spin drawing procedures to ensure the necessary degree of molecular alignment in the product (Kerkam et al., 1991, Carmichael and Viney, 1999). This knowledge is often used during artificial silk production, and after coagulation procedures the fibers are drawn to a certain length and most often allowed to dry in extended position. This procedure can be performed in a coagulant, in water or in air and repeated to optimize the mechanical properties of the fiber. This has also been combined with temperature treatments (Lazaris et al., 2002, Seidel et al., 2000, Bogush et al., 2008).

Electrospinning have also been employed when spinning recombinant spidroins dissolved in HFIP. As the name implies, the procedure involves extrusion of the protein feedstock in an electric field. The procedure has most often been used to spin fiber mats or mesh-works, and single fibers are not obtained (Stephens et al., 2005, Bogush et al., 2008). Moreover, electrospinning spidroin analogs in HFIP solutions has shown to induce α-helix structures, compared to β-sheets, which is the predominant conformation of the native proteins in the silk and believed to give the fiber its strength (Stephens et al., 2005).

1.9.1 Regenerated silk

Using a microfabricated wet-spinning apparatus, *B. mori* silk dissolved in HFIP to a concentration of 2.5% (w/w) was initially spun into a methanol coagulation bath. The formed fibers were drawn, soaked in methanol over night and annealed in vacuum at 40°C for 1h. An increase in β-sheet content was found with increasing draw ratio, as indicated by solid-state NMR spectroscopy. The mechanical properties varied significantly among produced fibers, with the best having a maximum strength approaching that of native *B. mori* silk (Liivak et al., 1998).

Using the same solvent, protein concentration and spinning approach, spider dragline silk from *N. clavipes* has been regenerated. The fibers where extruded in acetone coagulation baths and subjected to post-spinning draw and subsequent drying. The produced fibers varied in diameter between 20 and 80 μm. Alanine stretches in the produced fibers adopted mainly α-helical conformation and the fibers were shown to be very brittle. However, upon water treatment, an increase in the fraction of alanine residues taking on a β-sheet conformation was observed, as judged by NMR spectroscopy (Seidel et al., 1998).

A similar study from the same research group provided some optimizations to the process of regenerating *N. clavipes* dragline, and various post-spinning procedures were employed (different draw ratios, water-treatment and annealing in vacuum at 100°C). It was observed that if traces of water were included in the acetone coagulation bath together with post-spinning drawing, the tensile strength and stiffness could be enhanced. Fibers that had been in contact with water during the post-spinning processing displayed a general inelastic deformation of up to 100% before failure. Post-spinning draw increased the fraction of alanine residues adopting β-sheet conformation and the degree of β-sheet crystals in the fiber. Water was suggested to facilitate protein crystallization and to stabilize post-spinning draw processes (Seidel et al., 2000).

Additional studies intended to structurally and mechanically characterize regenerated dragline silk, produced by drawing filaments from aqueous silk solutions (Shao et al., 2003). Dragline from *N. edulis* was dissolved in buffer containing 8 M guanidine-HCl and gel-filtrated using deionized water, which gave an aqueous solution containing 0,08% w/w silk protein. A needle was used to draw a fiber from the surface of the solution where silk proteins were enriched and self-assembled. Secondary structure analysis of the proteins in the regenerated fiber suggested predominantly β-sheet conformation with random coil as the subordinate structure. It was suggested that the poor mechanical properties obtained for the regenerated silk were due to its formation by a self-assembling process of molecular chain aggregation instead of the complex liquid crystal spinning of the native silk. Moreover it was stated that the initial dissolving of the fiber could have denatured the proteins and altered their native conformations (Shao et al., 2003). This might also have a profound effect on protein-

protein interactions, and hence on the mechanical properties of regenerated silk filaments.

1.9.2 Recombinant spider silk

A lyophilized 58 kDa spidroin analog, representing the repetitive part of the MaSp2 spidroin from *N. clavipes*, was dissolved in formic acid at 2-4 mg/ml and forced through a 23 gauge needle into methanol to form a solid fiber. No further characterization except microscope examination of the fiber was conducted (Lewis et al., 1996).

One promising attempt in replicating the dragline silks extraordinary properties involved wet-spinning of a recombinantly produced dragline-protein analog in a urea-containing aqueous solution (Lazaris et al., 2002). A cDNA clone, coding for the repetitive part together with the C-terminal domain of the ADF-3 gene from the spider *A. diadematus* was expressed in BHK cells and resulted in the production of a 60 kDa protein. This protein was continuously produced from stably transfected cells in a hollow-fiber reactor. The protein was enriched by precipitation with ammonium sulfate, redissolved in aqueous buffer and purified by ion exchange chromatography. Fibers were spun from aqueous buffers containing urea with a recombinant ADF-3 protein concentration of 10-28% (w/v). Fibers with appreciable mechanical properties had either been single drawn in 70-80% methanol or double-drawn, first in methanol and a second time in water. Fibers displaying the best mechanical properties were typically derived from solutions with more than 23% protein concentration and had been subjected to high draw ratios reaching up to 5 times the fiber's original length. The drawing-procedure resulted in a decrease in fiber diameter with a concomitant increase in molecular orientation, as judged by microscopy measurements and increased birefringence. The water solubility of the proteins after precipitation enabled an aqueous based spinning procedure. The protein solubility was suggested to arise from including the hydrophilic C-terminal domain in the construct design (Lazaris et al., 2002).

Similar variants of wet-spinning procedures have, since these pioneering experiments, been employed and Table 3 summarizes some of the existing results from mechanical testing of regenerated silk and fibers from recombinantly produced spider silk analogs.

Table 3. *Properties of artificial silk*

Source	Protein	C-terminal domain	Size (kDa)	Fiber production	Spin-dope concentration	Post-spinning treatment	Stiffness E (MPa)	Tenacity/ Strength σ_{max} (MPa)	Strain at break ε_{max} (%)	Ref
Regenerated silk, *N. edulis*				Pulled from aqueous solution	0.08% (w/w)	No	~6,000	110-140	10-27	b
Regenerated silk, *N. clavipes*				Wet spinning HFIP	2.5% (w/w)	Coagulation bath and draw	1,000–8,000	10-320	<100	c
Mammalian cells	Partial ADF-3	Yes	60	Wet spinning Aqueous urea	10-28% (w/v)	Coagulation bath and draw	13,000[a] (43-111 gpd[d])	260[a] (1.8-2.3 gpd[d])	43-60	d, a
Yeast (*P. pastoris*)	*N. clavipes* MaSp1 & *N. madagascariensis* MaSp2, consensus repeats	No	94-113	Wet spinning LiCl/formic acid or NaNCS/acetate	>25%	Coagulation bath and draw annealing at 100°C	nd	100-150	5-15	e
Bacteria (*E. coli*)	*A. aurantia* MaSp2, consensus repeats	No	63-71	Wet spinning HFIP	10-12%	Coagulation bath	0.04-44	2-50	1.5-19	g
Bacteria (*E. coli*)	Composite Flag/MaSp-protein, consensus repeats	No	58-62	Pulled from aqueous solution	nd	No	1,100–1,700	29-50	19-34	f
				Wet spinning HFIP	25-30% (w/v)	Coagulation bath	760-930	10-29	2-4	

nd = not determined

[a]To facilitate comparison, tenacity values expressed in grams per denier are converted to Pa, as described in Kubik (2002)

[b]Shao *et al.* (2003), [c]Seidel *et al.* (2000), [d]Lazaris *et al.* (2002), [e]Bogush *et al.* (2008), [f]Teulé *et al.* (2007), [g]Brooks *et al.* (2008)

2 Present investigation

2.1 Aim

The primary aim of this thesis was to establish a recombinant expression system for large-scale, environmentally friendly, production of spider silk protein analogs for the intended manufacturing of biocompatible and biodegradable materials. In order to accomplish a production method for materials based on recombinant spider silk proteins, structural and behavioral characterization of expressed proteins is needed. Moreover, for production of materials, used in biomedical applications, purification of proteins and manufacturing procedures require minimized addition of non-physiological chemicals.

2.2 *Euprosthenops australis*

The choice of spider for the current work, with biomaterial applications in mind, fell on *Euprosthenops* for a number of reasons. First of all, it has been observed that the dragline silk of these spiders contract less than 20% in an aqueous environment (Shao and Vollrath, 1999), which could be due to the relatively high β-sheet content in the proteins of the fiber core (Shao et al., 1999b). In materials envisioned for biomaterial applications, supercontraction often is an unappreciated trait. Dragline silk from *Euprosthenops* sp. is approximately 2 μm in diameter (Shao and Vollrath, 1999) and one of the strongest and least extendible (1.5 GPa and 17%, respectively) of all dragline silks tested (Denny, 1980, Madsen et al., 1999, Gosline et al., 1999, Savage and Gosline, 2008a). Moreover, the dragline of *Euprosthenops* sp. displays a yielding point higher than for most other

draglines, meaning that it remains elastic even at high stress and does not irreversible deform (Madsen et al., 1999). In addition, the relatively large size of the spiders facilitated the dissection of major ampullate glands, used for subsequent mRNA isolation and construction of cDNA libraries. The spiders used in this study were all female and their identity as *E. australis* (Simon, 1898) was confirmed by Astri and John LeRoy from the Spider Club of South Africa.

The spider *Euprosthenops australis* belongs to the family Pisauridae, commonly called Nursery web or Fishing spiders, but contrary to what the latter name implies, the spider is terrestrial and often found in dry, arid bush landscapes on the African continent. *E. australis* produces a flat sheet web in low vegetation, approximately 1-3 m long and 1-1.5 m wide that lasts several months (Madsen et al., 1999). The web progressively narrows towards the ground, forming a funnel escape that leads into the vegetation, and sometimes further down into a silk-lined animal burrow, providing a safe and rapid retreat. The flat sheet web produced by *E. australis* have no direct adhesiveness or entanglement function, so the spiders have to rely on awareness and speed to instantly capture their pray, intercepted by the web. The genera *Euprosthenops* and *Euprosthenopsis* are similar in appearance and sometimes confused with each other, but contrary to species of the *Euprosthenopsis* genera, which occupies the top of its web, *Euprosthenops* spiders are larger, with a body length of approximately 3 cm, and is often found hanging inverted below the robust web. The prosoma of *E. australis* is covered with fine white hairs, the opisthosoma is yellow and the slender legs are striped in black and silver (Figure 6). All pisaurids construct a round white egg case that is carried under the body, held in the chelicerae (jaws), which causes them to assume a tiptoe stance. Just before the eggs are due to hatch, the female constructs a nursery web around the egg case. This is attached to the vegetation with a supporting web around it, see http://www.biodiversityexplorer.org/arachnids/spiders/pisauridae/ (2008-11-18).

Figure 6. Female *Euprosthenops australis* clinging vertically in her sheet-shaped web while carrying an egg case (photo: A. Rising).

2.3 Results and discussion

2.3.1 Transient expression of a major ampullate spidroin 1 gene fragment from *Euprosthenops* sp. in mammalian cells (I)

Spidroin analogs have successfully been expressed in mammalian cells and it has been suggested that, compared to microbial expression systems, the eukaryotic transcriptional and translational machinery might be better equipped to express spidroin genes (Lazaris et al., 2002).

In order to establish a recombinant expression system for the production of spider silk proteins, African green monkey kidney (COS-1) cells were transiently transfected with a vector construct containing a gene-segment coding for a repetitive part of MaSp1 from *Euprosthenops* sp. According to western blot analysis on cell-lysates, proteins of correct size were expressed, corresponding to the spidroin analog with and without leader peptide.

Compared to the expression of a positive control, it is apparent that the levels of produced dragline silk protein were low. The low expression levels observed are likely associated with the restricted amino acid usage in the production of spidroin proteins. It its possible that the tRNA-pools of the COS-1 cells are insufficient to allow high-level spidroin expression. The repetitiveness of the genes could also lead to secondary structure formations in the transcripts that impair full translation, leading to truncated proteins. However, size heterogeneity in the protein population due to ribosome processing errors would not be detected by western-blot since the detection tag is located in the C-terminal. In addition, low copy numbers of transfected constructs would also lead to low expression levels. Due to the low solubility of spider silk analogs, recombinant expression is often associated with aggregation of the target protein. The low levels detected by western blot could therefore also be a result of aggregated protein that is not possible to dissolve during normal SDS-PAGE procedures.

The expression of a partial spidroin corresponding to MaSp1 in cultured insect cells, invariably led to intracellular aggregations that was only possible to dissolve in harsh solvents (Huemmerich et al., 2004b). In previous studies, where recombinant spidroin analogs were successfully recovered, the proteins comprised the non-repetitive C-terminal domain, which was suggested to contribute to the solubility of the proteins (Lazaris et al., 2002). Connecting the repetitive region of MaSp1, expressed in this study, to the non-repetitive C-terminal domain could result in increased solubility of recombinant spider silk protein.

Since the aim of this study was to establish a recombinant expression system for production of recombinant spidroins for material construction and structural characterization, it was concluded that an alternative expression system, which could offer high protein yields had to be employed. The gene sequences for intended use should be small enough to facilitate bacterial expression without displaying significant instability.

2.3.2 Macroscopic fibers self-assembled from recombinant miniature spider silk proteins (II)

Because of the attractive mechanical properties of *Euprosthenops* dragline silk, a cDNA library was constructed from mRNA extracted from *Euprosthenops australis* major ampullate glands. By screening the library with a cDNA probe originated from *Euprosthenops* spiders of undefined subspecies a 3.8-kb clone was isolated. It coded for a 1207 amino acid fragment of MaSp1, containing 68 alanine- and glycine-rich blocks and a non-repetitive C-terminal domain.

The repetitive sequence of *E. australis* MaSp1 was analyzed and a frequently occurring motif was observed, consisting of four poly-Ala/Gly-rich tandem repeats, with a central turn. This motif, followed by the non-repetitive C-terminal domain, was chosen for expression in *E. coli*. In order to increase the solubility and facilitate purification, the miniature spidroin was expressed as a fusion protein comprised of thioredoxin/His-tag/S-tag/thrombin cleavage site/miniature protein. The fusion protein was easily purified by immobilized metal ion affinity chromatography and remained stable for weeks at 8°C. Protein yields of up to 40 mg/L shake-flask culture were obtained with more than 90% purity.

Upon enzymatic release from the fusion partner, the miniature spidroin polymerizes into macroscopic fibers. The fiber formation occurred at the air-liquid interface along the tube and was not dependent on changes in the environmental conditions such as reduction of pH or altered ion-concentrations. The only factor needed to promote fiber formation was the wagging of the reaction tube that might create elongational flow, thereby aligning the molecules. The mechanical properties of the fibers compared favorably to mammalian tendons (see Table 2) and fibers made from regenerated spider silk (see Table 3), with tensile strengths of approximately 0.2 GPa. The initial stiffness was about 7 GPa, which is similar to that of native dragline silk (Table 2). When analyzed by CD and X-ray diffraction, the fibers displayed similar structures to those of native spider silk fibers. SEM further indicated a substructure of tightly aligned fibrils.

Native dragline silk has shown to facilitate proper attachment and proliferation of nerve-tissue cells (Schwann cells) *in vitro*, without any observed toxic effect (Allmeling et al., 2006). This is in accordance to our study, where the recombinant fibers supported growth and adherence of human embryonic kidney (HEK) cells.

Hybrid constructs composed of a corresponding *Euprosthenops* repetitive part covalently linked to a *Nephila* C-terminal domain also formed fibers, indicating that the fiber forming potential of this motif is robust. However, successively reducing the length of the repetitive region led to a concomitant decline in fiber forming ability, and attempts to form fibers from the repetitive part separated from the C-terminal domain only resulted in amorphous aggregates. This suggests a crucial role for the C-terminal domain in the assembly process of spidroins and the formation of silk fibers. Since non-denatured miniature spidroins can spontaneously form fibers *in vitro*, this study indicates that the primary structure and conformation of the spidroins comprises the information needed to allow fiber formation. It also shows that only a small part of the full-size spidroin, consisting of four poly-Ala/Gly-rich blocks and the C-terminal domain, is sufficient to form macroscopic fibers.

Others have attempted to produce a 67 kDa recombinant spider silk protein, derived from a MaSp2 consensus sequence coupled to the non-repetitive C-terminal domain. Efforts to purify a measurable quantity of this protein failed, and it was found that the protein was sequestered in inclusion bodies. The incorporated C-terminal domain with its disulphide forming cysteine was suggested to be the reason for the inclusion body formation (Brooks and Lewis, 2004). However, the protein was not coupled to any solubility tag, such as thioredoxin, upon expression. By doing so, inclusion body formation might have been prevented. Paper II clarifies the advantage of producing the target protein fused to a solubility tag in a bacterial expression system.

Attempts have been made to increase solubility and control self-assembly of recombinant spidroin analogs by introduction of molecular switches in the protein design (Winkler et al., 1999, Winkler et al., 2000, Szela et al., 2000). Incorporation of methionine residues flanking poly-alanine blocks allowed for polymerization upon reduction with β-mercaptoethanol. In the reduced state, the methionines are hydrophobic, which allowed for β-sheet formation. Solubility was promoted by oxidation with phenacyl bromide that precluded β-sheet formation due to a combination of increased bulkiness and hydrophilicity at the sulfoxide side chain (Szela et al., 2000). However, for this system to work, the use of harsh chemicals was needed. Another approach was to introduce phosphorylation sites N-terminally positioned relative to the poly-alanine blocks. Phosphorylation was intended to hinder β-sheet assembly and improve solubility due to a combination of

increased bulkiness and charge-charge repulsion, and dephosphorylation was expected to have the opposite effect. However, in this case it was not reported if the self-assembly capacity of the spidroin analogs was retained after the gene-sequence alterations (Winkler et al., 2000).

Recently, other groups have produced fibers from recombinant spider silk analogs in aqueous solution, and they also stress the importance of preservation of the original secondary structures of recombinant silk proteins that might be critical for the resulting mechanical properties of the fiber (Teulé et al., 2007). However the mechanical properties of such fibers were low and varied considerably (Table 3). The fiber forming techniques used differed from that presented in this thesis, and the synthetic protein was based on the MaSp2 sequence, whereas in II the fiber forming proteins are derived from the MaSp1 sequence and from a different spider. Moreover, in II the proteins comprise the C-terminal domain, found to be important in assuring fiber formation. The incorporation of the C-terminal domain might also have a positive effect on the mechanical properties. In agreement with our results, others have also suggested an important role of the C-terminal domain in fiber assembly (Ittah et al., 2006).

In attempts to closer imitate the intricate spinning process of the spider, environmental changes that are thought to occur in the native silk production pathway, have been mimicked in the *in vitro* spinning procedure of fibers from recombinant dragline protein analogs using a microfluidic device (Rammensee et al., 2008). The two protein analogs are derived from consensus sequences of the structural proteins (ADF-3 and ADF-4) constituting the dragline of *A. diadematus* (Huemmerich et al., 2004a). eADF3 (106 kDa) comprises a stretch of the repetitive part and the non-repetitive C-terminal domain, whereas eADF4 (48 kDa) consists only of the repetitive part from the corresponding protein. The two protein analogs used, displayed different fiber forming propensities, where eADF3 formed fibers when pH was decreased from 8 to 6, and only under the influence of elongational flow. Fiber formation started at a protein concentration of 20 mg/ml and according to FTIR microscopy, produced fibers contained mainly β-sheet. Under the conditions investigated, eADF4 did not form fibers by itself and only irreversible aggregated to spherical particles. However, when mixing the two analogs at differing ratios prior to spinning, resulting fibers contained both protein analogs. Since only the non-repetitive C-terminal domain in eADF3 contains charged residues, it was suggested that the necessity of pH change likely indicates the importance of

the C-terminal domain for fiber assembly. However, fibers produced using this process are minute, approximately 100-200 μm long and have a heterogeneous appearance. These fibers have not been subjected to mechanical testing (Rammensee et al., 2008). In contrast, we have shown that fiber formation *in vitro* is not dependent on conformational changes as a result of lowered pH. However, discrepancies between the studies might be explained by differences in the primary structures and whether the proteins have been denatured during purification or not.

To summarize, in II a novel method for the efficient recombinant production of a soluble miniaturized spidroin under non-denaturing conditions is presented. This mini-spidroin can be processed under physiological-like conditions to form fibers with favorable mechanical and cell-compatibility properties, without the use of denaturing spinning procedures or coagulation treatments.

2.3.3 Structural properties of recombinant nonrepetitive and repetitive parts of major ampullate spidroin 1 from *Euprosthenops australis*: implications for fiber formation (III)

How spiders manage to store silk proteins, highly susceptible to aggregation and with the main purpose to polymerize into a solid fiber, at concentrations up to 50% (w/v), is still largely an enigma. Neither is the process fully elucidated, whereby the spider converts these proteins from a soluble form to an insoluble fiber. How the proteins are affected by the observed environmental changes (*i.e.* pH reduction and changes in salt composition) that do occur in the native fiber production pathway is important to clarify in order to successfully mimic the production using recombinantly produced analogs. These investigations are difficult to conduct in a reliable manner, using native silk dope, since the proteins are highly sensitive to shear forces and prone to aggregate upon handling.

In III, for the first time, all representative motifs of MaSp1 have been recombinantly produced under non-denaturing conditions, allowing investigation of the effect of changes in the environmental conditions. How factors such as temperature, pH and salt concentrations affect structure, stability, and conformational transitions of recombinant N- and C-terminal domains, a repetitive region, and combinations thereof was studied. The four motifs expressed in *E. coli* were the *E. australis* MaSp1 N- and C-

terminal domains, a fiber forming motif (4RepCT), comprised of four tandem poly-Ala/Gly-rich repeats followed by the C-terminal domain, and the repetitive part by itself (4Rep). Fiber formation and aggregation propensities were analyzed under various conditions.

Investigated salt concentrations or pH-conditions had no observed effect on the terminal domains, in regard to secondary structure changes, aggregation and/or fiber formation. However, 4Rep and 4RepCT aggregates during prolonged incubation at high concentrations (300 mM) of phosphate, both as sodium and potassium salts. Moreover, a change in the tertiary structure of 4Rep and 4RepCT was observed at these phosphate concentrations. 4Rep displays helical conformations, but seem to lack a stable folded structure. The major secondary structure of 4RepCT is α-helical, and as 4Rep, it adopts a β-sheet structure upon heating. The N- and C-terminal domains have a predominantly α-helical structure and both form homo-dimers at physiological pH. The terminal domains were more temperature-stable, and changes in the secondary structures occurred at higher temperatures, compared to 4Rep and 4RepCT. The N-terminal domain loses its structure when the temperature is raised to 90°C but regains most of its α-helical structure when the temperature is lowered to 20°C. The C-terminal domain displays α-helical structure up to 80°C, and at 90°C the structure is irreversible altered to β-sheets. The low content of aromatic residues in the C-terminal domain probably precluded detection of any change in tertiary structure by near-UV CD spectroscopy upon heat treatment. The reduction of a dimer-stabilizing disulphide in the C-terminal domain lowers the temperature stability but do not prevent the dimerization.

The N-terminal domain was efficiently expressed in bacteria and high yields could be recovered from the soluble fraction. Furthermore, it was observed that the N-terminal domain was stable for weeks at ambient temperatures and could be concentrated to more than 200 mg/ml without forming aggregates. From these results, it was suggested that the N-terminal could function as a solubility-enhancing domain for spidroins. It has previously been suggested that the N-terminal domain displays high conservation between spidroins from different species due to a general role in the structural and/or functional properties of the dragline silk (Rising et al., 2006, Ayoub et al., 2007). Contrary to the C-terminal domain, the incorporation of the N-terminal domain in the protein design is not a

requirement for the *in vitro* fiber formation presented here. This might imply a function more associated to protein solubility than fiber assembly.

4Rep forms minute fibrils and aggregates, whereas 4Rep coupled to the C-terminal domain, as in 4RepCT, forms macroscopic fibers. The same applies when the two constructs are heat-denatured. 4Rep aggregates and 4RepCT forms small fibers. These observations suggest that the C-terminal domain, covalently linked to 4Rep, favors ordered polymerization over unordered aggregation of the repetitive region. Therefore, the C-terminal domain seems to be important for proper fiber formation and could have the same function in native spidroins.

Dual functionalities have also been proposed where the C-terminal is suggested to be important for the solubility during storage, and promote precipitation of spidroins in the process of fiber production. It was concluded that shifts in the behavior and function of the C-terminal might be triggered by environmental changes (Sponner et al., 2005c). A region of high hydrophobicity in the C-terminal domain, stretching approximately 20 residues, has been suggested to form an amphipatic α-helix that is highly conserved between silks examined. One of the most conserved residues in the C-terminal domain is a Cys, located just N-terminally of the hydrophobic stretch (Challis et al., 2006, Sponner et al., 2005c). In addition to disulfide bridge formation, it was suggested that this part could mediate contacts through hydrophobic interactions (Sponner et al., 2005c). Moreover, the Cys is directly followed by a conserved, negatively charged amino acid (most often Asp), which was proposed to be involved in the formation of an intra-molecular salt bridge with an Arg in a neighboring α-helix within the C-terminal domain (Ittah et al., 2007). The C-terminal domain analyzed in III contains two conserved glutamic acid residues, one of them succeeding the disulphide-forming Cys. However, the secondary structure of the C-terminal domain is unchanged even at pH 2. The N-terminal domain is the most charged of the domains, with positive and negative residues, quite evenly distributed. However, the secondary structure of the N-terminal domain is likewise unaffected at pH 2-9. It is possible that lowering the pH, or altered ion compositions during the fiber production pathway weakens expulsion forces and facilitates alignment of spidroins for subsequent polymerization.

The terminal domains have been postulated to take part in forming multi-molecular complexes in the form of micelles, in order to facilitate the

high protein concentration observed in the gland and prevent premature aggregation (Jin and Kaplan, 2003, Bini et al., 2004). Based on the fact that only subtle structural changes in the individual representative modules constituting the MaSp1-protein of *E. australis* were seen (III), one could argue that the effects on the protein dope due to alterations in ion composition and lowering of pH to 6.3 might affect the integrity of proposed multi-molecular complexes.

Even though interactions between the N- and C-terminal domains could occur in order to organize the soluble protein complexes in the gland, direct analyses do not confirm such interactions. When the terminal domains were mixed, no difference in CD spectra could be disclosed when compared with the calculated combination of the two individual spectra. It is therefore reasonable to believe that no major interactions between the domains that lead to structural changes are prevalent. In addition, when the N-terminal domain was loaded onto a column with bound HisTrxHis-4RepCT, no retardation could be observed. Based on the observation that both terminal domains form homo-dimers, the formation of domain-connected protein multimers, that could have implications on protein solubility and/or fiber formation, is plausible. These interactions might be altered by changes in the environmental conditions (*i.e.* ion-exchange, pH and water removal), and together with shear forces induce dissociation of the multi-molecular complexes.

The increased level of aggregation observed for 4Rep and 4RepCT, during prolonged incubation with phosphate could be due to salt-induced hydrophobic interactions. Studies on small recombinant variants of ADF-3 and ADF-4 have also shown this effect of phosphate on aggregation and fiber polymerization (Exler et al., 2007, Huemmerich et al., 2004a, Rammensee et al., 2008) where high phosphate concentrations were a requirement for fiber formation. However, in III, high phosphate concentrations did not seem to affect fiber formation, which might be explained by the constant movement of the solution that lead to shear forces, compared to aggregation that was observed under non-shear conditions. Moreover, fiber formation occurs faster than the observed effect of phosphate on aggregation. The fact that fibers of 4RepCT are formed at phosphate concentrations of 500 mM further implies a quite robust fiber production system.

The effect of cationic ions, promoting silk protein structure transitions have further been demonstrated where films made from dilute *N. senegalensis* silk dope were treated with increasing concentrations of KCl. The analyzed films, indicated formation of β-sheet together with a decrease of random coil and/or helical structures (Chen et al., 2002a). K^+-ions have also been shown to promote the formation of nano-fibrils from dilute solutions of spidroin prepared directly from the protein stored in the ampulla (Chen et al., 2002b). In III however, no effect on structure or aggregation imposed by high KCl or NaCl concentration was seen.

In our hands, recombinant fiber formation readily occurs under physiologic-like conditions without increasing salt-concentrations or lowering pH, with elongational flow probably being the predominant factor for fiber formation. It has also been shown that fibers are formed through pulling silk from dilute aqueous solutions (Shao et al., 2003) with no change in the environmental conditions. This would indicate that the shear forces, mechanically denaturing and aligning the molecules, is the foremost factor during the final stages of native fiber formation. However, changes in the environmental conditions might facilitate this process.

The limited effect in III of lowered pH and high salt-concentrations on protein structure and behavior might reflect the usage of recombinant modules, instead of full-length proteins. It is plausible that these environmental changes have a stronger effect *in vivo* during the processing of full-size spidroins that can display interactions not possible in our investigation. In addition, one cannot exclude the possibility of other factors, yet undiscovered, that have a profound effect on native fiber formation.

2.3.4 Engineered disulfides improve mechanical properties of recombinant spider silk (IV)

Large-size silk proteins is a trait conserved through evolution in both spiders and insects and is suggested to be an important factor in contributing impressive mechanical properties to these biopolymer fibers (Bini et al., 2004, Ayoub et al., 2007). However, due to the low solubility of spidroins and instability of corresponding genes in expression hosts, recombinant production have only been able to provide spidroin analogs representing minute parts of the native silk protein.

In IV, amino acid altering mutations have been introduced into the sequence of a fiber forming motif, 4RepCT, consisting of four poly-Ala/Gly-rich tandem repeats followed by the C-terminal domain, in order to allow for covalent multimerization leading to the formation of large protein-entities. The aim was to increase the mechanical properties of recombinant spider silk fibers. Moreover, by replacing the conserved cysteine in the C-terminal with a similar sized amino acid, the importance of this Cys for dimerization and for fiber formation was investigated.

Three mutants were constructed from the 4RepCT gene sequence (CC1, CC2 and S) by site-directed mutagenesis. Upon expression, the target protein was N-terminally fused to a His/thioredoxin/His-tag that enabled recovery from the soluble fraction and swift affinity purification. Since it is generally accepted that intermolecular connections occur through stacking of β-sheets formed by poly-Ala stretches, disulphide-forming cysteines were introduced into poly-Ala segments. In CC1, two central alanines were altered to two adjacent cysteines in the most N-terminal poly-Ala block. In CC2, the two central alanines in the most C-terminal Ala-block were substituted with two neighboring cysteines. It became apparent upon expression of these two mutants that the location of the mutations had profound effect on the solubility of the proteins. CC1 was found soluble, whereas CC2 was sequestered in the insoluble fraction. Purification under reducing conditions did not increase the recovery of CC2. The possibility is that the incorporated cysteines in the fourth and most C-terminally poly-Ala block interact with the closely located, natively occurring cysteine in the C-terminal domain, and thereby becoming insoluble. Alternatively, by locating the cysteines into the fourth poly-Ala block, they might become too available for intermolecular disulphide formation, which could lead to CC2 insolubility. Moreover, the cysteines in CC1 reside close to the folded and bulky thioredoxin-tag that might sterically hinder intermolecular disulphide formation and thereby facilitate CC1 solubility.

Under non-reducing SDS-PAGE, S migrates as a monomer, where as 4RepCT and CC1 migrate a dimers. However, when analyzed by size exclusion chromatography under non-denaturing conditions, all three constructs migrate as dimers. This indicates that disulphide formation between C-terminal domains in not a prerequisite for dimer formation, but might function to stabilize the quaternary structure.

4RepCT, CC1 and S presents similar secondary structures in soluble form with a dominating α-helical conformation. S displayed somewhat lower temperature stability than 4RepCT that might be attributed to the eliminated capacity to form disulphide bridges between C-terminal domains. However, the three constructs demonstrate no difference in fiber forming propensities. This suggests that disulphide mediated dimerization in not a requirement for *in vitro* fiber formation by miniaturized spidroins. That S forms fibers without possibility for disulphide formation in the C-terminal domain is in agreement with III, where 4RepCT formed fibers under reducing conditions. Noteworthy is that most minor ampullate spidroins lack cysteines in their C-terminal region (Sponner et al., 2004). However, the high conservation of this cysteine residue between species indicates that it exerts an important function for dragline spidroins. The fibers made from the different constructs, formed as in II and III, have the same macroscopic- and microscopic appearance, in wet and dry state, respectively. Fibers, vigorously suspended in 2% SDS-buffer all displayed a typical β-sheet structure.

In order to facilitate intermolecular disulphide formation for CC1, fibers were formed or post-treated under redox conditions. When examining the properties of fibers made from CC1, 4RepCT was used as a control. In order to obtain homogenous fibers, possible to use for tensile testing, they were air-dried in a fixed, straight state. The different redox conditions did not lead to any difference in micro- or macroscopic morphology between the fibers, or between fibers made from different constructs.

The mechanical properties of the different fibers were surveyed by conducting tensile tests. During testing, the majority of the fibers broke at the point of fixation. This may be caused by imperfect alignment of the fibers with the direction of tension. The fibers can also been adversely affected by the force applied at the grip point when fixated. This is likely to result in an underestimation of the maximum stress- and strain values that might have been displayed by the fiber under optimal conditions. However, by determining the initial modulus, as a measure of stiffness, we obtained reliable values.

Fibers made from CC1 had higher initial modulus, with statistical significance, than fibers made from the control construct, 4RepCT. The maximum stress tolerated before breakage was also significantly higher for all fibers made from CC1, except for fibers treated with 10% of DMSO. CC1

fibers made in 3mM GSH/0.3mM GSSG seemed to have a higher initial modulus compared to other CC1-fibers, but the difference did not reach statistical significance. No difference in strain tolerance before breakage was observed between CC1- and 4RepCT fibers, while S fibers broke at lower extensions than 4RepCT. The initial modulus and maximum stress, however, did not differ significantly between 4RepCT- and S fibers.

To evaluate to what extent intermolecular disulphides had formed during and after fiber formation, fibers were dissolved in formic acid and analyzed by SDS-PAGE under reducing and non-reducing conditions. CC1 fibers showed bands corresponding to tetramers that disappeared upon reduction, indicating that cysteines introduced in the most N-terminally located poly-Ala block could form intermolecular disulphides. Fibers formed in various ratios of GSH/GSSG displayed additional bands corresponding to penta- and hexamers. However, it seems that only a fraction of the CC1 spidroins form high-molecular species through disulphide formation via introduced cysteines, since the majority of the proteins are in a dimeric form mediated by disulphides connecting the C-terminal domains. CC1-fibers treated with oxygen or 10% DMSO after formation did not display any additional bands corresponding to higher molecular species, other than tetramers.

Attempts have been made to vary extensibility properties of fibers produced from spidroin analogs by altering the numbers of the postulated elasticity motif GPGXX (Teulé et al., 2007). Even though a correlation was seen between the number of GPGXX-motif and the extensibility of the fibers, the strength and the stiffness was low and the fibers displayed significant variations in mechanical properties (Table 3). In II, it was shown that the mechanical properties of 4RepCT fibers could be improved by post-draw procedures that probably increase alignment of molecules. However, in order to achieve comparable fibers in a controlled manner, with as little variations as possible, post-draw procedures was not included in this study. The linear appearance of the stress strain curves for all constructs, lacking the typical yielding point (see figure 5) seen when testing native dragline silk, is indicative of a brittle material. The lack of yielding point might be due to the relatively short molecule chain that limits polypeptide entanglements.

The fact that CC1 fibers display significantly higher initial modulus than 4RepCT implies that stiffness is at least partly affected by increased interconnecting forces in β-sheet crystals formed by poly-Ala blocks. The

initial modulus and maximum stress tolerated by S fibers, without disulphide connected C-terminal domains, did not differ significantly from 4RepCT fibers. Together with the results from the superior CC1 fibers, this suggests that stiffness of fibers based on the 4RepCT construct relies mainly on molecular connections in the repetitive region, and in particular on interactions occurring in β-sheet crystals.

CC1 fibers formed under redox conditions seemed to result in more prominent disulphide formation and high-molecular species than treating fibers after formation in an oxidizing milieu. Post-treatment might be ineffective in generating intermolecular disulphides if the polypeptide chains are locked in a position where the cysteines are not accessible for interaction.

The larvae of the fly *Chironomus tentans* produces silk spun under water to construct feeding and pupation tubes. The silk comprises proteins found to be rich in cysteines that are suggested to form intermolecular disulphides (Wellman and Case, 1989). Analogs modeled after tandem repeats found in these silk proteins have been recombinantly produced in bacteria. However, it is found that the four cysteines in the protein analogs preferably form two intramolecular disulphide bonds. When the proteins were allowed to refold after being reduced and denatured, intramolecular disulphides were again formed. It was noted, however, that as the protein concentration increased, a concomitant increase in intermolecular disulphides were formed. It was suggested that during native silk formation, intramolecular disulphide formation would facilitate protein solubility, and as the protein concentration increases, intermolecular disulphide bonds would be favored (Smith et al., 1995).

It is possible that by further optimizing the oxidizing conditions during fiber formation of CC1, or by increasing the protein concentration, the disulphide dependent polymerization might be elevated with a concomitant increase in the mechanical properties of produced fibers.

Paper IV presents the first study where the mechanical properties of fibers made from recombinant spidroin analogs have been increased by genetic engineering, introducing covalent bonding options.

3 Concluding remarks and future perspectives

For 400 million years, the spider's fiber production mechanism has evolved to extreme refinement. Every part in the fiber production pathway is finely tuned and optimized in regard to each other, in order to produce a silk exactly matching its purpose. The spider produces high-performance biopolymers under ambient temperatures and pressures using only water as solvent, which is something man is not able to reproduce. In the industrial production of synthetic high-performance polymers, high temperatures and noxious solvents are used (Kerkam et al., 1991). Production costs and the manufacture time will increase with every additional chemical or process step introduced. Fore large-scale industrial production of artificial spider silk, where biomedical applications are envisioned, a "green" approach should be strived for where the need for harsh chemicals in the production process is abolished. In addition to lowering the production costs, it will also reduce the risk of adverse side effects in biological systems, or in the nature in general.

When planning to produce artificial spider silk the final use has to be considered, in order to chose the right production path. Does the final product require a material with superior mechanical properties, similar to that of dragline silk, or is properties such as biocompatibility and biodegradability more important?

If the intended application demands a material of supreme mechanical properties, the literature implies that a large protein size is imperative. Large-size silk proteins is a feature independently conserved through evolution in both spiders and insects, hence stressing its importance in the process of making a biological high-performance polymer (Bini et al., 2004).

It is therefore suggested that a complete silk gene, with full representation of the N- and C-terminal regions, the intervening repetitive sequence, and the transitions among these domains, should markedly improve recombinant silk performance (Ayoub et al., 2007). However, trials in regenerating fibers from dissolved silk have not been successful in reproducing the mechanical properties. This implies that large protein size alone will not suffice, and that a physiologic fiber-spinning process has to be employed where non-denatured spidroins in their natural conformations, comprising all essential parts is used, in order to mimic the properties of the dragline. A complete deciphering of the different and crucial components of the spiders spinning procedure would allow for successful benign spinning.

One could imagine that recombinant expression of full-size spidroins will pose problems. Full-size spidroin genes in current expression systems will most probably display instability, and together with translation errors reduce the recovery of the full-length protein. Moreover, the highly shear sensitive full-size spidroins would likely impose problems during purification procedures. Significant aggregation would be anticipated, requiring denaturing purification procedures.

It has been observed that during recombinant expression of spidroin analogs, the length of the individual repeat is important. The longer the repeated unit is, the more stable it will be (Fahnestock and Irwin, 1997). With this in mind, alternative spider silk proteins might be better suited as templates when designing genes for recombinant expression, which could lead to a more homogenous product and allow for larger proteins to be expressed. Aciniform silk is one of the toughest silks made by the orb-web spiders and is used to swath prey and to construct egg cases (Gosline et al., 1986, Hayashi et al., 2004, Hu et al., 2006). A gene coding for a protein, closely matching the amino acid content of the aciniform gland has been isolated (Hayashi et al., 2004). The predicted protein, aciniform spidroin 1 (AcSp1) contains more than 14 homogenized repeats, with each ensemble repeat consisting of 200 amino acids, and a non-repetitive C-terminal domain of 99 amino acids. Long poly-alanine and glycine-rich subrepeats, characteristic of dragline spidroins, are poorly represented in AcSp1. This silk protein, due to its long repeats and restricted use of alanine and glycine, compared to dragline spidroins, might represent an attractive alternative for recombinant expression.

An ideal expression system would allow for the recovery of recombinant full-size spidroins with correct secondary and tertiary structures, and with accurate higher order conformations, such as the postulated multi-molecular complexes. For this to be accomplished a milieu corresponding to the native storage environment of spidroins should be strived for and all parts of the spidroin, critical for correct folding and for achieving good mechanical properties, should be incorporated in the protein design. Native dragline silk is composed of two similar but distinct proteins (MaSp1 and MaSp2) comprising endogenous motifs that are postulated to give the dragline its unique mechanical properties. Incorporating both protein constituents in the fiber design could enhance the mechanical properties, more closely resembling the native dragline. However, if denaturing procedures are allowed for in the intended application and the required mechanical properties of the material are met, the protein constituents, conformations or size may be of inferior significance.

Perhaps the most promising future for recombinant spidroins lies in their use for biomedical applications, where biocompatibility and biodegradability is imperative. Applications for tissue regeneration and drug delivery could be envisioned, for which it is probably not necessary to reach the native dragline silk's mechanical properties.

Results presented in this thesis describe novel methods to produce recombinant miniature spidroins and macroscopic fibers under physiological-like conditions without the use of denaturing agents. Fiber production through self-assembly can be controlled and does not require spinning procedures. Production of this material through bacterial expression can allow for cost effective large-scale production.

Even though fibers produced in this manner display respectable mechanical properties, there are probably ways to enhance them even further.

- The introduction of optimized spinning procedures could positively alter the mechanical properties, partly by facilitating better alignment of the protein molecules.
- Post spinning treatments, such as post-formation draw and high-temperature annealing could be introduced, also to enhance molecular alignment and increase protein-protein interactions.

- The use of recombinant variants of both protein constituents of the native dragline (MaSp1 and MaSp2) in the fiber formation process.
- The use of recombinant spidroins comprising all described motifs (*i.e.* N-terminal domain, repetitive region and the C-terminal domain)
- By incorporating the N-terminal domain in the protein design, it might be possible to include longer segments of the repetitive region and still recover the protein in soluble form. The formation of multi-molecular assemblies might arise that would allow high solubility and protein concentration. If fibers are formed from such a construct, enhanced mechanical properties are anticipated.

This thesis describes for the first time the successful recombinant production of all representative parts of a major ampullate spidroin, used to elucidate the structural transitions of spidroins that might occur due to environmental changes during native silk spinning. Moreover, it is possible to create antibodies targeting the N-terminal domain to determine if it is incorporated into the dragline or not. This would further shed some light on spidroin processing and fiber formation, and the necessity of incorporating the N-terminal domain in the construct design when producing recombinant spidroin analogs.

In this thesis, it is shown that through altering the genetic sequence, coding for a miniaturized spider silk protein, it is possible to enhance the mechanical properties of self-assembled macroscopic fibers. By introducing options for intermolecular covalent bonding, the molecules can associate into larger protein-entities, which lead to enhanced mechanical properties. This is accomplished without changing the over-all capacity for fiber formation, which implicates a fiber formation process that likely would allow for incorporation of other functional motifs. Besides the advantages of spider silk being biodegradable and biocompatible, the possibility to design recombinant spidroins used in biomaterials tailored after the needs of specific tissues have been recognized. The potential of modified recombinant spider silk has been proven by the introduction of functional groups into the amino acid sequence, such as RGD for promoting cell attachment (Bini et al., 2006), or covalently linking functional groups to preassembled films made from native or recombinant silk material (Sofia et al., 2001, Huemmerich et al., 2006). Recombinant spidroin analogs have been

processed to form nanofiber-based hydrogels (Rammensee et al., 2006), with possible applications in tissue engineering, as well as microcapsules for potential use in drug-delivery systems (Hermanson et al., 2007). A pH-responsive recombinant silk-inspired polymer self-aggregates in water at low pH and may be of interest for biomedical applications, such as coating of surgical implants or pH-responsive controlled drug release (Werten et al., 2008).

By modifying the polymerization conditions, films, foams or gels of the presently studied recombinant miniature spidroin can be produced. This, in combination with the introduction of functional groups into the amino acid sequence, may allow the development of specialized matrices for regenerative medicine.

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Acknowledgements

The work presented in this thesis was carried out at the Department of Biomedical Sciences and Veterinary Public Health and the Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences. The work was supported by a grant (G5RD-CT-2002-00738) from the European Commission.

I would like to take the opportunity to thank the persons that in one way or another have contributed in the process of making this thesis, colleagues as well as friends and family:

Wilhelm, my supervisor. Thank you for providing me with this opportunity to take part of the world of spider silk. No matter if we threw ourselves down the ski slope, almost drowning in swamps looking for spiders or showing students how to party on the "finlands-ferry", we always had loads of fun. It has been an adventure!
Jan, my first co-supervisor. Thank you for taking in two stray PhD-students and for all the nice group dinners and activities. But most importantly, thank you for showing patience, providing scientific input and sharing your knowledge.
My, my second co-supervisor, a steroid injection for the project with a brilliant career as a scientist ahead. Don't you have 24-hour days as the rest of us? Thank you for pulling things together.
Kerstin, would anything work without you? In the end, my work depended on our early coffee breaks. I'm glad you took your responsibility. It has been a pleasure working with you.
Piotr, the labs "McGyver". Give him a peanut and gear stick and he will build you an incubator. Thank you for being that reassuring, relaxed guy. "No matter what happens today, the sun will still go up tomorrow".

Göran, always someone you can call. Thank you for scientific advice and friendship.
Margareta and **Armando**, thank you for your patience and supervision during my rookie years.
Tommy, thank you for caring and looking out for us in the beginning of the project.
Martin and **Anne-Sofie**, for always being there to help out when it was needed.
Professors and senior researchers, **Gunnar**, **Staffan**, **Liya**, **Sara** and **Elena**, thank you for providing an inspiring and creative atmosphere at the department.
Ingemar, a scientific role model. Thank you for sharing your immense knowledge and your captivating travel experiences.

Anna, my soon-to-be PhD-sister, who thought that you and I would write ourselves a book! Knowledge can take you far, but together with persistence it will take you all the way. Thank you and your lovely family for your friendship and support, it all means a lot. **Åsa**, the woman behind the woman. I'm looking forward to coffee-dates during our parental leave.
Anh-Tri, you are a genuine person and a true friend in all its essence. Ten years of hard-core friendship. We must celebrate, tequila? I can't wait for you to move back home and get me into shape. My three girls and I miss you.
Micke, why do good friends have a tendency to move far away! It's annoying. I guess I will have a hard time luring you back home.
Chris and **Camilla**, you are so easy to like. I hope you are well "over there", but I also hope that you miss Sweden and considering to migrate back. I promise more parties and wake-boarding.

Charlotte and **Hanna**, you have given me so much during these years. Thank you for the support and encouragement at work, but also for all the joyful social gatherings. **Petter**, I'm looking forward to do all these fatherly things during our paternity leave, I know Charlotte is a little bit worried.
Fredrik, bring the cigars to the party! Can I count on you when I have to shape up this saggy student body of mine next year, squash?
Jenny, my old room mate, always that caring and comforting person that you want to have in your back pocket when things get tough. You have such adorable children and I really enjoy **Sergio's** cooking!
Maria, BMC has its own little veterinary sun, always contagiously positive. Research needs people like you, but so does the rest of the world as well.

Glareh, the new member in the spider family. Your contagious laughter instantly cheers you up and it is hard not being happy when you are around.
Anna and **Linus**, living in the same city it is a shame that we don't see each other more often. I feel responsible for arranging a dinner. Consider it done.
Magnus and **Ingela**, I'm jealous of your house and impressed with you finishing it! This "glögg-träff" at "första advent" must become a tradition.
Michael, you are as eager and intelligent as you are friendly and polite. You will have no trouble finishing your PhD-studies.
Lotta, for your mellow personality, relaxed attitude and for always taking time to talk. I must come by and see the little one, congratulations!
Siwei, you are always so generous and positive with a constant smile on your face.
Urmimala and **Partha**. You saved my Sundays in the end. Thank you for always being so nice and caring.
Louise, if you have second thoughts abut science, you could make a fortune as the new "mat-Tina" with your winning personality and cooking!

The old gang with their respective partners: **Frida** and **Ulf**, **Cecilia** and **Niklas**, and **Jenny** and **Carl**. Thank you for all the parties and laughter, and for always encouraging and reassuring me with your experience. It means a lot.
My new room mates! **Hanna**, a pity you did not stay that long. You were an asset, both socially and scientifically. **Sara**, my personal coffee companion and computer-wiz "reboot the computer Stefan!". I will remember that. **Mona**, you could get me relaxed in the most stressed thesis-writing moments with your warm and comforting personality.
Ida, **Elin**, **Tiago**, **Annette**, **Jay**, **Beata**, **Fabio**, **Hanan**, **Osama**, **Sue**, **Ren** and **Carl-Olof**, for always keeping the mood up in the lab and in the red sofa. I will miss you all.

The Guys in alphabetic order ;-) **Alexander**, **Anders**, **Daniel**, **Fredrik**, **Johan**, **Jonas**, **Nicklas**, **Niklas**, **Mårten**, **Per**. Hey, I'm out of the cave! Thank you for always being there even though it is too seldom we see each other. The yearly skiing trip, all the activities and life-supporting parties is something I cherish. You are the best.
Anders and **Frida**, even though we live far apart we are always close. I value our friendship and the few times we see each other.
Jesper and **Line**, I'm so glad that you borrowed my key to the apartment.

Thank you for being such dear and concerned friends, and for producing dashing potential partners for my daughters! I'm longing for dinners, sauna, "bada isvak" and "äta rökt sidfläsk med morakniv".

Last, but most importantly, I would like to thank my family:

Agnethe and **Lars**, my parents-in-law, thank you for always making me feel welcomed and loved. **Mats**, my brother-in-law, I miss spending time with you and you family. I can even imagine going out on the ice during this Christmas, all though I know that we will not catch any fish.

To my brothers and true friends, I'm so proud of you and your wonderful families.
Tomas, thank you for your warm, considerate and loving personality, and for always being happy and fun to be around.
Jesper, you are always so thoughtful and caring. Thank you for your insight and good advises that I always listen to.
Petrus, my youngest brother who is not that little any more. You have been away to long now. It is time to come home. Your smart and trouble-free mind together with your relaxed attitude towards life and odd sense of humor makes you very hard to dislike.

Ulrika, **Maria** and **Maria**, Thank you for being the best sisters I never got and for my wonderful nieces. Fantastic, more is yet to come!

Mum and **Dad**, thank you for always believing in me and for your constant love and support. I obviously made a wise decision when choosing parents.

Hanna, my partner in life and to whom I owe everything. Thank you for our two daughters, for your love and patience, and for making me a better person.

Ida and **Stina**, my two angels who make life so easy to live. I love you and your mother always.