
Recombinant spider silk proteins

The present invention is directed to recombinant spider silk proteins, nucleic acids, coding for these recombinant spider silk proteins, as well as hosts suitable for expressing those nucleic acids. Furthermore, the present invention is directed to a method of aggregation of spider silk proteins and the use of the proteins in the field of biotechnology and/or medicine and other industrial fields, in particular in the manufacture of automotive parts, in the aircraft construction, in the processing of textiles and leather, as well as in the manufacture and processing of paper, cosmetics, food, electronic devices, drug delivery and the like.

In this application, the following abbreviations will be used:

NR, non-repetitive; Ap^r, ampicillin resistance gene; IPTG, Isopropyl- β -D-thiogalactosid; GdmCl, guanidinium chloride; GdmSCN, guanidinium thiocyanate; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)aminomethane; CD, circular dichroism; rep-proteins, repetitive proteins; Da, Dalton; cps, counts per second; MRW, mean residue weight; n.d., not determined.

Spider silks are protein polymers that display extraordinary physical properties (1). Among the different types of spider silks, draglines are most intensely studied. Dragline silks are utilized by orb weaving spiders to build frame and radii of their nets and as lifelines that are permanently dragged behind. For these purposes high tensile strength and elasticity are required. The combination of such properties results in a toughness that is higher than that of most other known materials (1;2). Dragline silks are generally composed of two major proteins whose primary structures share a common repetitive architecture (3;4).

Variations of a single repeat unit, which can comprise up to 60 amino acids, are iterated several times to represent the largest part of a dragline silk sequence. These repeat units comprehend a limited set of distinct amino acid motifs. One motif found in all dragline silk repeat units is a

block of typically 6 - 9 alanine residues. In silk threads several poly-alanine motifs form crystalline β -sheet stacks leading to tensile strength (5;6).

Glycine rich motifs such as GGX or GPGXX adopt flexible helical structures that connect crystalline regions and provide elasticity to the thread (7).

Additionally, all investigated dragline silk proteins comprise regions at their carboxyl termini that display no obvious repetition pattern (non-repetitive- or NR-regions). So far no function could be assigned to these regions in the final thread.

Silk assembly *in vivo* is a remarkable process. Spider dragline silk proteins are stored at concentrations up to 50 % (w/v) (8) in the so-called major ampullate gland. Although a "dynamic loose helical structure" has been proposed for the proteins within the major ampullate gland (8) more recent data suggests a random coil conformation for the proteins of the so called A-Zone, which represents the largest part of the gland (9;10). The highly concentrated protein solution forms the silk dope (spinning solution), which displays properties of a liquid crystal (11-13).

Thread assembly is initiated during a passage of the dope through the spinning duct accompanied by extraction of water, sodium and chloride (14;15). At the same time the concentrations of the more lyotropic ions potassium and phosphate are increased and the pH drops from 6.9 to 6.3 (14-16). Assembly is finally triggered by mechanical stress, which is caused by pulling the thread out of the spider's abdomen (17).

For several purposes natural silk threads can not be used directly, but have to be dissolved and reassembled into other morphologies such as films, foams, spheres, nanofibrils, hydrogels and the like.

Most investigations concerning films made from silk proteins have been performed with silk fibroin, the main protein component of the silk from the silkworm *Bombyx mori*. Silk fibroin films can be cast from aqueous solutions or from solutions containing hexafluoroisopropanol (HFIP), formic acid, and trifluoro acetic acid. In solution silk fibroins tend to adopt helical or

random coil conformations, depending on the solvent used. When cast into films, proteins either maintain the conformation of the soluble state or adopt a more β -sheet rich conformation. In most cases processing of the films with methanol leads to a further increase of β -sheet content and crystallinity. Besides silk fibroin, other silk proteins have also been employed to cast films. Vollrath and co-workers investigated films made of proteins extracted from major ampullate gland of the spider *Nephila senegalensis*. As-cast films mainly contained proteins in a random coil conformation when prepared from aqueous solution. Their structure changed into β -sheet upon addition of potassium chloride. Further, films have been made from a synthetic silk protein derived from the dragline silk protein MaSp1 of the spider *Nephila clavipes* using HFIP as solvent. In solution the protein adopted an α -helical structure changing to a more β -sheet rich conformation when cast into a film.

Unfortunately, the generation of functional film materials from natural silk fibroin is restrained by its amino acid sequence. Selective chemical modification of silk fibroin is only possible to a very limited extent due to the low abundance (<1.5%) of chemically reactive amino acid side chains that contain thiol, amino or carboxyl groups. Further, genetic modification within the natural host to alter the silk protein's and thus the film's properties is tedious.

While some structural aspects of spider silk proteins have been unravelled, still little is known about the contribution of individual silk proteins and their primary structure elements to the assembly process. Comparative studies of the two major dragline silk proteins of the garden spider *Araneus diadematus*, ADF-3 and ADF-4, revealed that, although their amino acid sequences are rather similar (4), they display remarkably different solubility and assembly characteristics: While ADF-3 is soluble even at high concentrations (18), ADF-4 is virtually insoluble and self-assembles into filamentous structures under specific conditions (unpublished results).

Scientific and commercial interest initiated the investigation of industrial scale manufacturing of spider silk. Native spider silk production is impractical due to the cannibalism of spiders, and artificial production has encountered problems in achieving both sufficient protein yield and quality thread-assembly. Bacterial expression yielded low protein levels, likely caused by a

different codon usage in bacteria and in spiders. Synthetic genes with a codon usage adapted to the expression host led to higher yields, but the proteins synthesized thereof showed different characteristics in comparison to native spider silks. Expression of partial dragline silk cDNAs in mammalian cell lines did yield silk proteins (e.g. ADF-3) that could be artificially spun into 'silken' threads, albeit as yet of inferior quality.

WO03060099 relates to methods and devices for spinning biofilament proteins into fibers. This invention is particularly useful for spinning recombinant silk proteins from aqueous solutions and enhancing the strength of the fibers and practicality of manufacture such as to render commercial production and use of such fibers practicable. Therein, it is disclosed to express spider silk proteins in mammalian cells, e. g. transgenic goat mammary gland cells.

Expression of authentic spider silk genes in bacterial hosts is - as mentioned above - inefficient (24) since some gene sections contain codons not efficiently translated in bacteria. In addition, gene manipulation and amplification by PCR is difficult due to the repetitive nature of silks. In order to investigate properties of spider silk proteins, cloning strategies have been employed using synthetic DNA modules with a codon usage adapted to the corresponding expression host. Synthetic genes were obtained which coded for proteins resembling the repetitive regions of spider silks (25-28). However, none of these protein designs included the carboxyl terminal NR-regions that are found in all dragline silks.

Therefore it is an object underlying the present invention to provide recombinant silk spider proteins having enhanced characteristics as, in particular, improved capability of being expressed in high yield and improved strength and flexibility, i.e. better quality. Furthermore, it is an object of the present invention to provide recombinant spider silk proteins, which can be conveniently expressed in already known expression systems. It is a further object of the invention to provide an improved method for the aggregation of spider silk proteins and a method for forming threads made of these proteins. Additionally, it is an object of the present invention to provide improved paper, textile and leather products. Additional objects are to provide new proteins and further materials based on spider silk proteins such as spheres, nanofibrils, hydrogels, threads, foams,

films for use in biotechnology, medicine, pharmaceutical and food applications, cosmetics, in electronic devices and for other commercial purposes.

These objects are solved by the subject-matter of the independent claims. Preferred embodiments are set forth in the dependent claims.

The present protein engineering approach, which provides recombinant spider silk proteins, comprising or consisting of synthetic repetitive spider silk protein sequences and/or authentic NR-(non repetitive) regions, reveals that proteins closely resembling authentic silk proteins can be produced at high yields. In particular, the bacterial expression system as well as the simple and cheap purification process provided herein, which can easily be scaled up, provides the basis for cost-efficient industrial scale production of spider silk-like proteins.

Spider silk proteins have mainly been investigated regarding their contribution to mechanical properties of the silk thread. However, little is known about the molecular mechanisms of silk assembly. As a first step towards characterizing this process, the inventors identified primary structure elements of the garden spider's (*Araneus diadematus*) major dragline silk proteins ADF-3 and ADF-4 that determine protein solubility. Further, the influence of conditions involved in mediating natural thread assembly on protein aggregation was investigated. Genes coding for spider silk-like proteins were generated using a newly developed cloning strategy, which is based on a combination of synthetic DNA modules and PCR-amplified authentic gene sequences. Comparing secondary structure, solubility and aggregation properties of the synthesized proteins revealed that single primary structure elements have diverse influences on protein characteristics. Repetitive regions representing the largest part of dragline silk proteins determined the solubility of the synthetic proteins, which differed greatly between constructs derived from ADF-3 and ADF-4. Factors, such as acidification and increase of phosphate concentration, which promote silk assembly *in vivo*, generally decreased silk protein solubility *in vitro*. Strikingly this effect was pronounced in engineered proteins comprising the carboxyl terminal non-repetitive regions of ADF-3 or ADF-4, indicating that these regions play an important role in initiating assembly of spider silk proteins.

According to a first aspect, the present invention is directed to a recombinant spider silk protein comprising

- a) one or more synthetic repetitive spider silk protein sequences, and / or
- b) one or more authentic non-repetitive spider silk protein sequences.

The term “synthetic repetitive sequence” as used herein is to be understood as a recombinant protein sequence, which can not be found in nature, which is, however, derived from repeat units, which are naturally occurring in spider silk proteins. As indicated above, those repetitive sequences comprise one or more single repeat units, which comprise up to 60 amino acids. The naturally occurring repeat units comprehend a limited set of distinct amino acid motifs. Those repeat units confer inter alia tensile strength and elasticity to the thread, which may be later on formed from the spider silk protein.

The different types of repeat units, which may be form the base for the synthetic repetitive sequence of the invention, will be explained in detail below.

The second component of the recombinant spider silk protein of the invention, which may be present in addition to the synthetic repetitive sequences or alone, comprises one or more authentic non-repetitive protein sequences. These non-repetitive sequences play an important functional role in thread assembly.

It is noted that in the present invention, also recombinant spider silk proteins are contemplated, which only comprise synthetic repetitive sequences. Although the recombinant proteins of the invention showing both components, i.e. synthetic repetitive sequences as well as authentic non-repetitive sequences, have a broader range of utility and can be yielded in higher amounts (see chapter Examples below), the recombinant spider silk proteins having only synthetic repetitive sequences included can be used for some specific applications.

These applications are – inter alia – automotive and aircraft parts, surface coatings, as well as wound closure systems and wound dressings. Or in other words, applications, in which no thread structures of spider silk proteins are required.

The term “authentic” as used herein means that the underlying nucleic acid sequences are isolated from their natural environment without performing substantial amendments in the sequence itself. The only modification, which is accepted to occur, is where the authentic non-repetitive nucleic acid sequence is modified in order to adapt said sequence to the expression in a host without changing the encoded amino acid sequence. Preferred sequences are NR3 (SEQ ID NO: 10; derived from ADF-3) and NR4 (SEQ ID NO: 11; derived from ADF-4). In both sequences, for more efficient translation, the codon AGA (Arg), which is rarely translated in *E.coli*, was mutated to CGT (Arg) using PCR mutagenesis.

Preferred authentic non-repetitive sequences of flagelliform proteins are the amino acid sequence and nucleic acid sequence of FlagN-NR (SEQ ID NOs: 31 and 32) and FlagC-NR (SEQ ID NOs: 33 and 34).

According to a preferred embodiment, the recombinant spider silk proteins of the invention generally are derived from spider dragline proteins from the spider's major ampullate gland and/or from proteins derived from the flagelliform gland.

According to a further preferred embodiment, the authentic non-repetitive sequences are derived from the amino terminal non-repetitive region (flagelliform proteins) and/or the carboxy terminal non-repetitive region (flagelliform and dragline proteins) of a naturally occurring spider silk protein. Preferred examples of those proteins will be indicated below.

It is generally preferred to select the dragline and/or flagelliform sequences from dragline or flagelliform proteins of orb-web spiders (Araneidae and Araneoids).

More preferably the dragline proteins and/or flagelliform proteins are derived from one or more of the following spiders: *Arachnura higginsii*, *Araneus circuli*, *Araneus diadematus*,

Argiope picta, Banded Garden Spider (*Argiope trifasciata*), Batik Golden Web Spider (*Nephila antipodiana*), Beccari's Tent Spider (*Cyrtophora beccarii*), Bird-dropping Spider (*Celaenia excavata*), Black-and-White Spiny Spider (*Gasteracantha kuhlii*), Black-and-yellow Garden Spider (*Argiope aurantia*), Bolas Spider (*Ordgarius furcatus*), Bolas Spiders - Magnificent Spider (*Ordgarius magnificus*), Brown Sailor Spider (*Neoscona nautica*), Brown-Legged Spider (*Neoscona rufofemorata*), Capped Black-Headed Spider (*Zygiella calyptrata*), Common Garden Spider (*Parawixia dehaani*), Common Orb Weaver (*Neoscona oxancensis*), Crab-like Spiny Orb Weaver (*Gasteracantha cancriformis (elipsoides)*), Curved Spiny Spider (*Gasteracantha arcuata*), *Cyrtophora moluccensis*, *Cyrtophora parnasia*, *Dolophones conifera*, *Dolophones turrigera*, Doria's Spiny Spider (*Gasteracantha doriae*), Double-Spotted Spiny Spider (*Gasteracantha mammosa*), Double-Tailed Tent Spider (*Cyrtophora exanthematica*), *Aculeperia ceropegia*, *Eriophora pustulosa*, Flat Anepsion (*Anepsion depressum*), Four-spined Jewel Spider (*Gasteracantha quadrispinosa*), Garden Orb Web Spider (*Eriophora transmarina*), Giant Lichen Orbweaver (*Araneus bicentenarius*), Golden Web Spider (*Nephila maculata*), Hasselt's Spiny Spider (*Gasteracantha hasseltii*), *Tegenaria atrica*, *Heurodes turrata*, Island *Cyclosa* Spider (*Cyclosa insulana*), Jewel or Spiny Spider (*Astracantha minax*), Kidney Garden Spider (*Araneus mitificus*), Laglaise's Garden Spider (*Eriovixia laglaisei*), Long-Bellied *Cyclosa* Spider (*Cyclosa bifida*), Malabar Spider (*Nephilengys malabarensis*), Multi-Coloured St Andrew's Cross Spider (*Argiope versicolor*), Ornamental Tree-Trunk Spider (*Herennia ornatissima*), Oval St. Andrew's Cross Spider (*Argiope aemula*), Red Tent Spider (*Cyrtophora unicolor*), Russian Tent Spider (*Cyrtophora hirta*), Saint Andrew's Cross Spider (*Argiope keyserlingi*), Scarlet *Acusilas* (*Acusilas coccineus*), Silver *Argiope* (*Argiope argentata*), Spinybacked Orbweaver (*Gasteracantha cancriformis*), Spotted Orbweaver (*Neoscona domiciliorum*), St. Andrews Cross (*Argiope aetheria*), St. Andrew's Cross Spider (*Argiope Keyserlingi*), Tree-Stump Spider (*Poltys illepidus*), Triangular Spider (*Arkys clavatus*), Triangular Spider (*Arkys lancearius*), Two-spined Spider (*Poecilopachys australasia*), *Nephila* species, e.g. *Nephila clavipes*, *Nephila senegalensis*, *Nephila madagascariensis* and many more (for further spider species, see also below). Most preferred, the dragline proteins are derived from *Araneus diadematus* and the flagelliform proteins are derived from *Nephila clavipes*.

In the context of this invention, it should be clear that a recombinant spider silk protein may not only comprise protein sequences from one species, but may also contain sequences derived from different spider species. As an example, the one or more synthetic repetitive spider silk protein sequences might be derived from one species, the one or more authentic non-repetitive spider silk protein sequences from another. As a further example, it is also possible to design a recombinant spider silk protein, which contains more than one type of a repetitive sequence, wherein the different types are derived from different species.

According to one preferred embodiment, the dragline protein is wild type ADF-3, ADF-4, MaSp I, MaSp II and the flagelliform protein is FLAG. The term ADF-3/-4 is used in the context of MaSp proteins produced by *Araneus diadematus* (*Araneus diadematus* fibroin-3/-4). Both proteins, ADF-3 and -4 belong to the class of MaSp II proteins (major ampullate spidroin II).

The silk fiber has crystalline regions of β -sheets interspersed with elastic amorphous segments similar to liquid crystalline polymers. These two segments are represented by two different protein classes, MaSp I (major ampullate spidroin I) and MaSp II (major ampullate spidroin II) coded by different genes.

In a further embodiment, the nucleic acid sequence provided is ADF-3 (SEQ ID NO:1) and/or ADF-4 (SEQ ID NO: 2), or a variant thereof.

It is noted that two different kinds of ADF-3 and ADF-4 coding sequences and proteins are contemplated in this invention: first, the already published sequence of ADF-3 and ADF-4 (herein: "wild type" sequence) and, second, a variant thereof, encoded by SEQ ID NO: 1 (ADF-3) and 2 (ADF-4). The wild type sequences were already published and are available under the accession numbers U47855 and U47856 (SEQ ID NO: 8 and 9).

Further spider silk proteins, which can be used in this invention (i.e. alone or in combination with further proteins) and their database accession numbers are:

spidroin 2 [*Araneus bicentenarius*]gi|2911272

major ampullate gland dragline silk protein-1 [Araneus ventricosus] gi|27228957
major ampullate gland dragline silk protein-2 [Araneus ventricosus] gi|27228959 ampullate
spidroin 1 [Nephila madagascariensis] gi|13562006
major ampullate spidroin 1 [Nephila senegalensis] gi|13562010
major ampullate spidroin 1 [Latrodectus geometricus] gi|13561998
major ampullate spidroin 1 [Argiope trifasciata] gi|13561984
major ampullate spidroin 1 [Argiope aurantia] gi|13561976
dragline silk protein spidroin 2 [Nephila clavata] gi|16974791
major ampullate spidroin 2 [Nephila senegalensis] gi|13562012
major ampullate spidroin 2 [Nephila madagascariensis] gi|13562008
major ampullate spidroin 2 [Latrodectus geometricus] gi|13562002

According to another preferred embodiment, the flagelliform protein is SEQ ID NO: 6 (Flag-N) and/or SEQ ID NO: 7 (Flag-C) or a variant thereof, which constitute novel sequences derived by the inventors.

However, also already known and published flagelliform sequences may be used herein, in particular the following:

Flagelliform silk protein partial cds [Nephila clavipes] gi|2833646

Flagelliform silk protein partial cds [Nephila clavipes] gi|2833648

In one preferred embodiment, the recombinant spider silk protein comprises one or more synthetic repetitive sequences containing one or more polyalanine containing consensus sequences. Those polyalanine sequences may contain from 6 to 9 alanine residues. See, for example SEQ ID NO: 1, containing several polyalanine motifs of 6 alanine residues.

Preferably, the polyalanine containing consensus sequence is derived from ADF-3 and has the amino acid sequence of SEQ ID NO: 3 (module A) or a variant thereof. Module A contains a polyalanine having 6 alanine residues. A further preferred polyalanine containing consensus sequence, derived from ADF-4, is module C (SEQ ID NO: 5), containing 8 alanine residues.

According to a further preferred embodiment, in the recombinant spider silk protein of the invention, the synthetic repetitive sequence is derived from ADF-3 and comprises one or more repeats of the amino acid sequence of SEQ ID NO: 4 (module Q) or a variant thereof.

In more general words, a synthetic repetitive sequence may also contain the general motifs: GGX or GPGXX, i.e. glycine rich regions. As mentioned above, these regions will provide flexibility to the protein and thus, to the thread formed from the recombinant spider silk protein containing said motifs.

It is noted that the specific modules for the synthetic repetitive sequence of the invention can also be combined with each other, i.e. modules (repeat units) combining A and Q, Q and C etc. are also encompassed by the present invention. Although the number of the modules to be introduced in the spider silk protein is not restricted, it is preferred to employ a number of modules of the synthetic repetitive sequence for each recombinant protein which number is preferably ranging from 5-50 modules, more preferably 10-40 and most preferably between 15-35 modules.

The synthetic repetitive sequence preferably comprises one or more of (AQ) and/or (QAQ) as repeat units. Even more preferred, the synthetic repetitive sequence is (AQ)₁₂, (AQ)₂₄, (QAQ)₈ or (QAQ)₁₆.

Whenever the synthetic repetitive sequence is derived from ADF-4, it may preferably comprise one or more repeats of the amino acid sequence of SEQ ID NO: 5 (module C) or a variant thereof, as mentioned above, wherein the overall synthetic repetitive sequence is C₁₆ or C₃₂.

Preferred embodiments for the complete recombinant spider silk proteins of the invention are (QAQ)₈NR3, (QAQ)₁₆NR3, (AQ)₁₂NR3, (AQ)₂₄NR3, C₁₆NR4 and C₃₂NR4 i.e. proteins which comprise or consist of said sequences.

It is noted that the above configuration of the synthetic repetitive sequence (using the A, Q and C system) also applies to all other repeat units disclosed above, for example all polyalanine containing sequences can be taken for A and/or C and all glycine rich sequences may be used as Q.

New modules for synthetic repetitive sequences derived from flagelliform sequences are modules K (SEQ ID NO: 35 and 36), sp (SEQ ID NO: 37 and 38), X (SEQ ID NO: 39 and 40), and Y (SEQ ID NO: 41 and 42):

The synthetic repetitive sequence also preferably comprises or consists of Y₈, Y₁₆, X₈, X₁₆, K₈, K₁₆.

Furthermore, it is also possible, to combine those sequences derived from ADF-3 and ADF-4 and Flag in one recombinant sequence.

As explained above, the amino acid sequences disclosed herein are not restricted to the exact sequences provided in the SEQ ID Nos. The amino acid sequences indicated herein also comprise variants. Thus, the amino acid sequences of the proteins of the present invention also encompass all sequences differing from the herein disclosed sequences by amino acid insertions, deletions, and substitutions.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids, preferably about 1, 2 or 3 amino acids. Amino acid additions typically are not more than 100, preferably not more than 80, more preferably not more than 50, most preferred not more than 20 amino acids, which are added on and/or inserted into the proteins of the present invention. It is noted that only those additions are contemplated in this invention, which do not negatively affect the desired characteristics of the proteins disclosed herein.

The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a protein using recombinant DNA techniques and assaying the resulting recombinant variants for activity. This does not require more than routine experiments for the skilled artisan.

The present invention is, according to a second aspect, directed to a nucleic acid sequence, coding for a recombinant spider silk protein as disclosed above. Preferred sequences coding for preferred proteins are SEQ ID NO: 12 (ADF-3), 13 (ADF-4), 14 (NR3), 15 (NR4), 16 (FLAG-NT), 17 (FLAG-CT), 32 (FlagN-NR), 34 (FlagC-NR).

The invention also encompasses variants of those nucleic acids. These variants are each defined as having one or more substitutions, insertions and/or deletions as compared to the sequences of SEQ ID NO: 12-17, 32 and 34, provided that said variants hybridize under moderately stringent conditions to a nucleic acid which comprises the sequence of SEQ ID NO: 12-17, 32 and 34, or provided that said variants comprise nucleic acid changes due to the degeneracy of the genetic code, which code for the same or a functionally equivalent amino acid as the nucleic acid sequence of SEQ ID NO: 12-17, 32 and 34.

The term "nucleic acid sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides. The terms "nucleic acid" and "polynucleotide" are used interchangeably herein to refer to a heteropolymer of nucleotides.

Stringency of hybridization, as used herein, refers to conditions under which polynucleotide duplexes are stable. As known to those of skill in the art, the stability of duplex is a function of

sodium ion concentration and temperature (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2nd Ed. (Cold Spring Harbor Laboratory, (1989)). Stringency levels used to hybridize can be readily varied by those of skill in the art.

As used herein, the phrase "moderately stringent conditions" refers to conditions that permit DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, more preferably about 85% identity to the DNA; with greater than about 90% identity to said DNA being especially preferred. Preferably, moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5 x Denhart's solution, 5 x SSPE, 0.2% SDS at 42°C, followed by washing in 0.2 x SSPE, 0.2% SDS, at 65°C.

According to a third aspect, a vector is provided which comprises the above mentioned nucleic acids. Preferably, an expression vector is provided, which comprises said nucleic acids. This expression vector preferably comprises one or more regulatory sequences. The term "expression vector" generally refers to a plasmid or phage or virus or vector, for expressing a polypeptide/protein from a DNA (RNA) sequence. An expression vector can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an amino-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

According to a preferred embodiment, the vector is a plasmid or a viral vector, which preferably is a baculovirus system or a vaccinia virus vector system. Further viral vector systems may also be used in this invention. From case to case, a modification of the vector may be needed. Examples for further viral vectors are adenoviruses and all negative-strand RNA-viruses, e.g. rabies, measles, RSV, etc.

According to a preferred embodiment, the vector is the cloning vector pAZL as defined in Figure 6 or in SEQ ID NO: 55, or a variant thereof as defined above. This vector is showing the following properties and advantages:

1. high amplification (higher than other cloning vectors)
2. allows controlled and seamless construction of synthetic genes (no other vector is known that provides this ability).

A fourth aspect of the invention comprises a host, which has been transformed with the vector as defined above.

The host may be a prokaryotic cell. In this case, *E.coli* or *Bacillus subtilis* are preferred.

Furthermore, the host may be a eukaryotic cell, preferably a mammalian cell, plant cell, yeast cell or an insect cell.

The mammalian cell preferably is a CHO, COS, HeLa, 293T, HEH or BHK cell.

It is also preferred to use a yeast cell as a host cell, which preferably is *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Candida albicans* or *Hansenula polymorpha*.

As insect cells Lepidoptera insect cells may preferably be used, more preferably cells from *Spodoptera frugiperda* and from *Trichoplusia ni*. Most preferably, the insect cell is a Sf9, Sf21 or high five cell.

One advantage of insect cell expression system, for example regarding bacterial systems, resides in the fact that the proteins produced are glycosylated, thereby being a target for degradation by microorganisms. This characteristic may be of importance, for example, in the field of medicine, whenever the silk proteins are intended for an *in vivo* use, in which biological degradation is

desired. This characteristic may in particular find application in suture materials and wound closure and coverage systems.

Whenever the host is a plant cell, the plant cell preferably is derived from tobacco, potato, corn and tomato.

According to a fifth aspect, a method of aggregation of spider silk proteins is provided, comprising the following steps:

- a) preparing a protein solution containing unoriented spider silk proteins as defined herein;
- b) exposing the solution prepared in a) to an aggregation trigger; and
- c) recovering the precipitated spider silk proteins.

Preferably, the spider silk proteins used in step a) are produced by transforming a suitable host as defined above with a vector or a nucleic acid disclosed herein, and expressing the spider silk gene under suitable conditions.

The aggregation trigger is preferably selected from acidification, preferably to a pH of about 1, potassium phosphate and mechanical stress, preferably rotating the protein solution and applying shearing forces. The triggering step turned out to be essential for performing the method of this invention.

It was surprisingly shown by the inventors that in particular the above mentioned trigger factors enhanced the aggregation of spider silk proteins, which is a highly wanted result in particular from an industrial point of view. Reference in this connection is made to the chapter „Results“ below, in which the influence of these trigger factors on the recombinant spider silk proteins of the invention is explained: the influence of each trigger factor may vary between the different recombinant spider silk proteins of this invention, however, it can be seen as a general concept that those trigger factors *in vitro* show an unexpectedly high influence on all recombinant proteins, which comprise the components of the present invention, i.e. repetitive and / or non-repetitive regions. Furthermore, it can be derived from the results provided herein that not a

single trigger factor, but also combinations of those may lead to the best way of aggregate spider silk proteins of the invention.

However, it should be noted that this method is not restricted to the spider silk proteins of the present invention, but can also be applied to all other spider silk proteins available, whether naturally occurring or synthetic.

The method further preferably comprises the step of spinning said proteins prepared in step a) or recovered in c) into filaments, nanofibers and threads by a suitable method.

For this purpose, spinning methods may be used, which are per se known in the art. For example, a dope solution of spider silk protein is extruded through a spinneret to form a biofilament. The resulting biofilament can be drawn or stretched. Whenever both crystalline and amorphous arrangements of molecules exist in biofilaments, drawing or stretching will apply shear stress sufficient to orient the molecules to make them more parallel to the walls of the filament and increase the tensile strength and toughness of the biofilament.

The dope solution may contain the recombinant silk proteins of the invention and/or authentic silk proteins from one or more spider species, or silk proteins from different silk-producing genera, for example, a mixture of silk proteins from spiders and *B. mori*. In the most preferred embodiments, the silk proteins are dragline and/or flagelliform silks from *N. clavipes* or *A. diadematus*, particularly the proteins MaSpI, MaSpII, ADF-3, ADF-4 and Flag. In alternate embodiments, the dope solution contains a mixture of silk proteins and one or more synthetic polymers or natural or synthetic biofilament proteins.

Preferably, the dope solution is at least 1%, 5%, 10%, 15% weight/volume (w/v) silk protein. More preferably, the dope solution is as much as 20%, 25%, 30%, 35%, 40%, 45%, or 50% w/v silk protein. In preferred embodiments, the dope solution contains substantially pure spider silk protein. In preferred embodiments, the dope has a pH of approximately 6.9.

By "dope solution" is meant any liquid mixture that contains silk protein and is amenable to extrusion for the formation of a biofilament or film casting. Dope solutions may also contain, in addition to protein monomers, higher order aggregates including, for example, dimers, trimers, and tetramers. Normally, dope solutions are aqueous solutions of pH 4.0-12.0 and having less than 40% organics or chaotropic agents (w/v). Preferably, the dope solutions do not contain any organic solvents or chaotropic agents, yet may include additives to enhance preservation, stability, or workability of the solution.

By "filament" is meant a fiber of indefinite length, ranging from nanoscale and microscopic length to lengths of a mile or greater. Silk is a natural filament, while nylon and polyester as an example are synthetic filaments.

Further information regarding how to spin spider silk protein fibers may be found in WO03060099 (Karatzas et al.), published July 24, 2003, which is incorporated herein by reference.

Furthermore, the spider silk proteins of the present invention may be provided as films or the like, i.e. as a spider silk protein product, for which a spinning step is not required.

For a more detailed description of the process of making films it is referred to chapter Examples.

Additionally, the method of the present invention may preferably include in step a) and/or c) a purification method, comprising exposing the expressed spider silk proteins to heat denaturation at 60-90, preferably 70-80 °C followed by addition of ammonium sulphate of 600-1400 mM, preferably 800-1200 mM.

As already explained above, the proteins/threads as defined herein may be used in the field of biotechnology and/or medicine, preferably for the manufacture of wound closure or coverage systems, suture materials for use in neurosurgery or ophthalmic surgery.

Furthermore, the proteins/threads may preferably be used for the manufacture of replacement materials, preferably artificial cartilage or tendon materials.

Additionally, the threads/fibers of the invention can be used in the manufacture of medical devices such as medical adhesive strips, skin grafts, replacement ligaments, and surgical mesh; and in a wide range of industrial and commercial products, such as clothing fabric, bullet-proof vest lining, container fabric, bag or purse straps, cable, rope, adhesive binding material, non-adhesive binding material, strapping material, automotive covers and parts, aircraft construction material, weatherproofing material, flexible partition material, sports equipment; and, in fact, in nearly any use of fiber or fabric for which high tensile strength and elasticity are desired characteristics. Adaptability and use of the stable fiber product in other forms, such as a dry spray coating, bead-like particles, or use in a mixture with other compositions is also contemplated by the present invention.

It is explicitly noted that the most preferred applications of the spider silk proteins of the present invention are in the manufacture and processing of clothing fabric (textiles) and leather, automotive covers and parts, aircraft construction materials as well as in the manufacture and processing of paper.

The recombinant spider silk proteins of the present invention may be added to cellulose and keratin and collagen products and thus, the present invention is also directed to a paper or a skin care and hair care product, comprising cellulose and/or keratin and/or collagen and the spider silk proteins of the present invention. Papers and skin care and hair care products, in which the proteins of the present invention are incorporated are showing improved characteristics, in particular improved tensile strength or tear strength.

Furthermore, the recombinant spider silk proteins of the invention may be used as a coating for textile and leather products, thereby conferring stability and durability to the coated product. The silk proteins in particular show applicability for coating leather products, since in this case, tanning and its negative effects for environment can be avoided or at least reduced.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The invention is now further illustrated by Examples and the accompanying drawings, which are showing the following:

Figure 1 Cloning strategy for constructing synthetic spider silk genes. **(A)** The cloning cassette comprised restriction sites required for module multimerization (*BsgI* and *BseRI*) and for excising assembled genes (*NcoI*, *BamHI*, *HindIII*). During gene construction the spacer region was replaced by modules and module multimers. **(B)** Site-directed connecting of two modules was accomplished by ligating two appropriate plasmid fragments. The vector's ampicillin resistance gene (*Apr*^r) was reconstituted. **(C)** Nucleotides required for linking two modules were confined within the first codon of each module. **(D)** Module multimers were connected like single modules resulting in controlled assembly of synthetic genes. **(E)** Amino acid sequences of designed silk modules were derived from dragline silk proteins ADF-3 and ADF-4.

Figure 2 Analysis of spider silk proteins. **(A)** T7-tags of recombinant silk proteins were detected after western blotting with an anti-T7-tag antibody. **(B)** Proteins were subjected to SDS-PAGE followed by silver staining. Due to weak staining of (AQ)₁₂ and (QAQ)₈ the contrast of the image was increased electronically. **(C)** Fluorescence emission spectra of purified C₁₆NR4 are shown with excitation wavelengths of 280 nm (straight line) or 295 nm (dotted line), respectively.

Figure 3 Secondary structure and temperature transitions of spider silk proteins. **(A)** CD-spectra of rep-proteins (straight lines), repNR-proteins (dotted lines) and NR-proteins (long dashes) were recorded at 20°C. **(B)** Mean residue weight (MRW) ellipticities of soluble spider silk proteins

were measured at 220 nm while heating synthetic silk proteins to 90°C (straight line), followed by cooling to 20°C (dotted line).

Figure 4 Aggregation of synthetic spider silk proteins. Aggregation of proteins was determined after incubation for 1 hour in buffer (control), in the presence of 300 mM NaCl, or 300 mM KCl, at pH 1 or in the presence of 300 mM potassium phosphate. Bars for proteins derived from ADF-3: light grey; from ADF-4: dark grey.

Figure 5 Cloning strategy for constructing synthetic flagelliform spider silk genes (see Figure 1). Single modules were connected to homo-multimeres (a) as well as hetero-multimeres (b). (c) shows the amino acid sequences of designed flagelliform silk modules derived from flagelliform silk protein (Flag) from *Nephila clavipes*.

Figure 6 is showing a restriction map of vector pAZL.

Figure 7: Assembly forms of spider silk proteins. **(A)** Spheres formed by C₁₆ visualized by scanning electron microscopy (SEM). **(B)** Nanofibrils formed by C₁₆NR4 visualized by atomic force microscopy (height information). **(C, D)** Microfibril formed by (AQ)₂₄NR3 investigated by SEM (C). For cutting the fibril and subsequent visualization of the cross section a focused Ga⁺ ion beam was used (D). **(E)** Foam generated from a (AQ)₂₄NR3 solution. **(F)** Foam generated from a C₁₆NR4 solution. **(G)** Crosslinked gel formed by C₁₆NR4 nanofibrils.

Figure 8: CD-spectra of synthetic silk proteins (AQ)₂₄NR3 and C₁₆ dissolved in 6 M guanidinium thiocyanate followed by dialysis against 5 mM potassium phosphate pH 8.0 (straight line) or dissolved in HFIP (dotted line).

Figure 9: C₁₆ film cast from a 2% w/v C₁₆ solution in HFIP.

Figure 10: CD-spectra of protein films made from (AQ)₂₄NR3 and C₁₆. Films were cast from a protein solution in HFIP directly on a plain quartz glass and analyzed by CD-spectroscopy (dotted line). The film was subsequently processed with 1 M potassium phosphate and re-

analyzed. Due to inaccuracies in defining the thickness of the films, $\square\Theta_{MRW}$ could not be determined.

Figure 11: Modification of C_{16} films cast from a HFIP solution and processed with potassium phosphate. (A) Efficient coupling of fluorescein (yellow colour) only occurred when the carboxyl groups of C_{16} were activated (+) using EDC. In contrast only little fluorescein bound to films without EDC activation (-). (B) Activity of coupled β -galactosidase was monitored using X-Gal as substrate. The occurrence of a blue precipitate indicated enzyme activity only on films that had been activated with EDC (+), while non-activated films only showed residual enzymatic activity (-).

Figure 12: AFM image of C_{16} nanofibers.

Figure 13: Hydrogels prepared of C_{16} nanofibers.

Figure 14: The stress/strain behavior of the crosslinked and non-crosslinked hydrogels at a concentration of 10 mg/ml.

Figure 15: Frequency dependence of the storage modulus (G') and loss modulus (G'') for both the crosslinked and non-crosslinked fiber networks at a concentration of 20 mg/ml.

Figure 16: Concentration dependence of the storage modulus at a frequency of 0.5 Hz for both the crosslinked and the non-crosslinked hydrogels. Both networks have storage moduli that are proportional to the concentration squared $[c]^2$.

EXAMPLES

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from Merck KGaA (Darmstadt, Germany) if not otherwise stated. Manipulation and modification of DNA was performed as described previously (19). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA) and ligase from Promega Biosciences Inc. (San Luis Obispo, CA, USA). DNA purification was performed using kits from Qiagen (Hilden, Germany). Synthetic oligonucleotides were obtained from MWG Biotech AG (Ebersberg, Germany). All cloning steps were performed in the *E. coli* strain DH10B from Novagen (Madison, WI, USA).

Construction of the cloning vector pAZL. A cloning cassette with cohesive ends complementary to ones generated by *Bgl*III and *Hind*III was created by annealing two synthetic oligonucleotides CC1 (GATCGAGGAGGATCCATGGGACGAATTCACGGCTAATGAAAGCTTACTGCAC) (SEQ ID NO: 18) and CC2 (AGCTGTGCAGTAAGCTTTCATTAGCCGTGAATTCGTC CCATGGATCCTCCTC) (SEQ ID NO: 19). Annealing was accomplished by decreasing the temperature of a 50 pmol/μl (each) oligonucleotide solution from 95°C to 20°C with an increment of 0.1°C/s. Mismatched double strands were denatured at 70°C followed by another temperature decrease to 20°C. After repeating the 20°C-70°C-20°C cycle ten times, ten additional cycles were performed with a denaturing temperature of 65°C. The resulting cloning cassette was ligated with a pFastbac1 vector (Invitrogen, Carlsbad, California, USA) digested with *Bgl*III and *Hind*III. Both restriction enzyme recognition sequences were destroyed upon this cloning step. The resulting new cloning vector was named pAZL.

Cloning of silk modules and NR-regions into the pAZL vector. Three amino acid modules derived from the dragline silk proteins ADF-3 and ADF-4 (Fig.1E) were back translated into a DNA sequence considering bacterial codon usage. Corresponding complementary DNA oligonucleotides A1 (TCCGTACGGCCCAGGTGCTAGCGCCGCAGCGGCAGCGGCTGGT GGCTACGGTCCGGGCTCTGGCCAGCAGGG) (SEQ ID NO: 20) and A2 (CTGCTGGCCAGAGCCCGGACCGTAGCCACCAGCCGCTGCCGCTGCGGCGCTAGCAC

CTGGGCCGTACGGACC) (SEQ ID NO: 21), Q1 (TCCGGGCCAGCAGGGCCCCGGGTCAACAGGGTCCTGGCCAGCAAGGTCCGGGCCAGCAGGG) (SEQ ID NO: 22) and Q2 (CTGCTGGCCCCGGACCTTGCTGGCCAGGACCCTGTTGACCCGGGCCCTGCTGGCCCCGGACC) (SEQ ID NO: 23), C1 (TTCTAGCGCGGCTGCAGCCGCGGCAGCTGCGTCCGGCCCCGGGTGGCTACGGTCCGGAAAACCAGGGTCCATCTGGCCCCGGGTGGCTACGGTCCGTGGCGGTCCGGG) (SEQ ID NO: 24) and C2 (CGGACCGCCAGGACCGTAGCCACCCGGGGCCAGATGGACCCTGGTTTTCCGGACCGTAGCCACCCGGGGCCGGACGCAGCTGCCGCGGCTGCAGCCGCGCTAGAACC) (SEQ ID NO: 25) were synthesized and annealed as described above and ligated with the pAZL vector digested with *BsgI* and *BseRI*. NR-regions of spider silk genes *adf-3* (gi|1263286) and *adf-4* (gi|1263288) (obtained from Prof. Gosline, Vancouver, Canada) were amplified by PCR using the following primers: NR3f (GAAAAACCATGGGTGCGGCTTCTGCAGCTGTATCTG) (SEQ ID NO: 26), NR3r (GAAAAGAAGCTTTCATTAGCCAGCAAGGGCTTGAGCTACAGATTG) (SEQ ID NO: 27), NR4f (GAAAAACCATGGGAGCATATGGCCCATCTCCTTC) (SEQ ID NO: 28) and NR4r (GAAAAGAAGCTTTCATTAGCCTGAAAGAGCTTGGCTAATCATTTG) (SEQ ID NO: 29).

For Flag sequences, the following primers and cassettes may be used:

PCR-Primer:

FLAG-N-chr-sense: (SEQ ID NO: 43)

5'- GAAAAACCATGGGCGAAAGCAGCGGAGGCGAT - 3'

FLAG-N-chr-anti: (SEQ ID NO: 44)

5'- GAAAAGAAGCTTTCATTAGCCTGGGCTGTATGGTCC - 3'

FLAG-C-chr-sense: (SEQ ID NO: 45)

5'- GAAAAACCATGGGTGCTTATTATCCTAGCTCGC - 3'

FLAG-C-chr-anti: (SEQ ID NO: 46)

5'- GAAAAGAAGCTTTCATTAGCCATAAGCGAACATTCTTCCTAC - 3'

Oligos for repetitive sequences from which cassettes were generated:

Module Y-(GPGGX)-ds: (SEQ ID NO: 47)

5'-
TCCGGGCGGTGCGGGCCCAGGTGGCTATGGTCCGGGCGGTTCTGGGCGGGGTGGCT
ACGGTCCTGGCGGTTCCGGCCCCGGGTGGCTACGG - 3'

Module Y-(GPGGX)-cs: (SEQ ID NO: 48)

5'-
GTAGCCACCCGGGCCCAGAACCGCCAGGACCGTAGCCACCCGGCCCAGAACCGCCCCG
GACCATAGCCACCTGGGCCCCGCACCGCCCCGGACC - 3'

Module sp-(spacer)-ds: (SEQ ID NO: 49)

5'-
TGGCACCACCATCATTGAAGATCTGGACATCACTATTGATGGTGCGGACGGCCCCGAT
CACGATCTCTGAAGAGCTGACCATCGG - 3'

Module sp-(spacer)-cs: (SEQ ID NO: 50)

5'-
GATGGTCAGCTCTTCAGAGATCGTGATCGGGCCGTCCGCACCATCAATAGTGATGTC
CAGATCTTCAATGATGGTGGTGCCACC - 3'

Module K-(GPGGAGGPY)-ds: (SEQ ID NO: 51)

5'-
TCCGGGCGGTGCTGGCGGTCCGTACGGCCCTGGTGGCGCAGGTGGGCCATATGGTCC
GGGCGGTGCGGGCGGTCCGTACGG - 3'

Module K-(GPGGAGGPY)-cs: (SEQ ID NO: 52)

5'-

GTACGGACCGCCCGCACCGCCCGGACCATATGGCCACCTGCGCCACCAGGGCCGT
ACGGACCGCCAGCACCGCCCGGACC - 3'

Module X-(GGX)-ds: (SEQ ID NO: 53)

5'-TGGCGCTGGTGGCGCCGGTGGCGCAGGTGGCTCTGGCGGTGCGGGCGGTTCCGG -
3'

Module X-(GGX)-cs: (SEQ ID NO: 54)

5'-GGAACCGCCCGCACCGCCAGAGCCACCTGCGCCACCGGCGCCACCAGCGCCACC
- 3'

PCR-products and pAZL vector were ligated after digestion with *NcoI* and *HindIII*. Cloning of synthetic modules as well as PCR-products resulted in the replacement of the cloning cassette's spacer, preserving the arrangement of its elements. For more efficient translation, the codon AGA (Arg), which is rarely translated in *E.coli*, was mutated to CGT (Arg) in NR3 and NR4 using PCR mutagenesis (19).

Construction of synthetic spider silk genes. Connecting of two gene fragments e.g. single modules, module multimers or NR-regions represented the basic step of the cloning strategy. For this purpose the pAZL vector, containing the designated 5'-terminal gene fragment was digested with *BsaI* and *BsgI*, while the vector comprising the 3'-terminal gene fragment was digested with *BseRI* and *BsaI* respectively (Fig.1B). Ligation of the appropriate plasmid fragments yielded the connecting of the two gene fragments and led to the reconstitution of the pAZL vector's ampicillin resistance gene (*Ap^r*) which facilitated identification of correct constructs.

For gene construction, single modules were first connected to yield repeat units (Fig.1D + Fig.5). These were gradually multimerized and optionally linked with NR-regions. Finally, synthetic gene constructs as well as NR-regions were excised from the pAZL vector with *BamHI* and *HindIII* and ligated with the bacterial expression vector pET21a (Novagen) likewise digested,

providing a T7-tag (MASMTGGQQMGR) (SEQ ID NO: 30) coding sequence (20). The fidelity of all constructs was confirmed by DNA sequencing.

Gene expression. All silk genes were expressed in the *E. coli* strain BLR [DE3] (Novagen). Cells were grown at 37°C in LB medium to an $OD_{600} = 0.5$. Before induction with 1 mM IPTG (Isopropyl- β -D-thiogalactosid), cells were shifted to 30°C in the case of (AQ)₁₂, (AQ)₁₂NR3, (QAQ)₈, and (QAQ)₈NR3 and to 25°C in the case of C₁₆, C₁₆NR4, NR3 and NR4 respectively. Alternatively cells were grown in a fermenter to an $OD_{600} = 40-50$ using complex media (21) and the fed-batch technique (22). Again, before induction with 1 mM IPTG cells were shifted to 25°C or 30°C, respectively. Cells expressing (AQ)₁₂, (AQ)₁₂NR3, (QAQ)₈, (QAQ)₈NR3, C₁₆ and C₁₆NR4 were harvested after 3-4 hours of induction while cells expressing NR3 and NR4 were harvested after 16 hours.

Protein purification. Cells were resuspended with 5 ml/g buffer containing 20 mM N-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) pH 7.5, 100 mM NaCl, 0.2 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 4°C for 30min. Cells were lysed by sonification using a HD/UW2200/KE76 ultrasonicator (Bandelin, Berlin, Germany) and genomic DNA was digested by incubating cell lysates with 0.1 mg/ml DNase I (Roche, Mannheim, Germany) and 3 mM MgCl₂ at 4°C for 60 min. Insoluble cell fragments were sedimented at 50,000×g and 4°C for 30 min. Soluble *E. coli* proteins of lysates containing (AQ)₁₂, (AQ)₁₂NR3, (QAQ)₈, (QAQ)₈NR3, C₁₆ and C₁₆NR4 were precipitated by heat denaturation at 80°C for 20 min while lysates containing NR3 and NR4 were heated to 70°C for the same length of time. Precipitated proteins were removed by sedimentation at 50,000×g for 30 min. Silk proteins, which remained soluble during heat denaturation, were precipitated with 20% ammonium sulphate (800 mM) ((AQ)₁₂, (AQ)₁₂NR3, (QAQ)₈, (QAQ)₈NR3, C₁₆ and C₁₆NR4) or 30% ammonium sulphate (1200 mM) (NR3 and NR4) at room temperature and harvested by centrifugation at 10,000×g for 10 min. Pellets of (AQ)₁₂, (AQ)₁₂NR3, (QAQ)₈, (QAQ)₈NR3, NR3 and NR4 were rinsed with a solution containing the same concentration of ammonium sulphate as used for precipitation and dissolved in 6 M guanidinium chloride (GdmCl). In contrast C₁₆ and C₁₆NR4 were washed with 8 M urea and dissolved in 6 M guanidinium thiocyanate (GdmSCN). All proteins were dialyzed against 10 mM NH₄HCO₃. Precipitates

formed during dialysis were removed by sedimentation at 50,000×g for 30 min and the remaining soluble silk proteins were lyophilized. Prior to analysis lyophilized protein was dissolved in 6 M GdmSCN followed by dialysis against appropriate buffers. Aggregates were removed by sedimentation at 125,000×g for 30 min. Protein concentrations were determined photometrically in a 1 cm path length cuvette at 276 nm using calculated extinction coefficients (Table 1) (23). Identity of proteins was confirmed by sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE; 10% Tris-Glycine gels for proteins > 20 kDa and 10 - 20% Tris-Tricine gels (Invitrogen) for proteins < 20 kDa) followed by blotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and detection using a mouse anti-T7 monoclonal antibody (Novagen, 1:10,000) as primary and anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, 1:5,000) as secondary antibody. Peroxidase activity was visualized using the ECL^{plus} western blot detection kit from Amersham Biosciences (Piscataway, NJ, USA).

Fluorescence. Fluorescence spectra were recorded on a FluoroMax Spectrofluorometer (Jobin Yvon Inc, Edison, NJ, USA). Spectra were taken using a protein concentration of 100 µg/ml in 10 mM Tris(hydroxymethyl)aminomethane (Tris) / HCl (pH 8.0) at room temperature. Integration time was 1 s, step size was 0.5 nm and band widths were 5 nm (excitation) and 5 nm (emission), respectively.

Secondary structure analysis. Far-UV circular dichroism (CD) spectra were obtained using a Jasco 715 spectropolarimeter equipped with a temperature control unit (Jasco International Co. Ltd., Tokyo, Japan). All spectra were taken at a protein concentration of 150 µg/ml in 5 mM Tris/HCl (pH 8.0) in a 0.1 cm path length quartz cuvette at 20 °C. Scan speed was 20 nm/min, step size was 0.2 nm, integration time was set to 1 s and band width was 1 nm. Four scans were averaged and buffer-corrected. Thermal transitions were analyzed with a heating / cooling increment of 1°C/min at 220 nm.

Solubility assay. To determine the maximal concentration of soluble proteins, a 1 mg/ml (= 0.1% (w/v)) solution in 10 mM Tris/HCl pH 8.0 was concentrated by ultra filtration using a 10,000 Da molecular weight cut off polyether sulfone membrane (Vivascience AG, Hannover, Germany).

At distinct intervals samples were taken from the solution until the protein started to precipitate. Samples were diluted in 10 mM Tris pH 8.0 to determine protein concentration photometrically.

Aggregation assay. All samples were adjusted to 1 mg/ml in 10 mM Tris/HCl pH 8.0. For testing ionic effects on silk protein aggregation, salts were added to final concentrations of 300 mM. The effect of acidification was investigated by adding HCl to a final concentration of 100 mM (pH = 1). All samples were incubated at room temperature for 1 hour. Protein precipitates were removed from all samples by sedimentation at 125,000×g for 25 min and the amount of the remaining soluble protein was determined photometrically. Since the sum of soluble and aggregated protein had to equal the initial amount of soluble protein, the percentage of aggregated protein could be calculated by subtracting the amount of soluble protein from the initially used amount of protein.

RESULTS

A cloning strategy for designing silk-like proteins. Expression of authentic spider silk genes in bacterial hosts is inefficient (24) since some gene sections contain codons not efficiently translated in bacteria. In addition, gene manipulation and amplification by PCR is difficult due to the repetitive nature of silks. In order to investigate properties of spider silk proteins, cloning strategies have been employed using synthetic DNA modules with a codon usage adapted to the corresponding expression host. Synthetic genes were obtained which coded for proteins resembling the repetitive regions of spider silks (25-28). Importantly, none of these protein designs included the carboxyl terminal NR-regions that are found in all dragline silks.

The inventors developed a seamless cloning strategy (29) that allowed controlled combination of different synthetic DNA modules as well as authentic gene fragments. The cloning vector pAZL was designed comprising a cloning cassette with a spacer acting as placeholder for synthetic genes, and recognition sites for the restriction enzymes *BseRI* and *BsgI* (Fig.1A). Since recognition and cleavage sites of these enzymes are 8 (*BseRI*) or 12 (*BsgI*) nucleotides apart, translation start and stop codons as well as additional restriction sites required for the excision of assembled genes could be positioned close to the spacer.

In a first cloning step the spacer region of pAZL was replaced by a synthesized DNA module (for module design see below). Subsequently two modules could be joined in a site-directed way (see materials and methods and Fig.1B). The complementary 3'-single strand extensions GG (sense) and CC (antisense) generated by cleavage with *BsgI* and *BseRI* were used for connecting two modules (Fig.1C). Thus the DNA sequence required to link two modules was confined to a glycine codon (GGX). Glycine is naturally abundant in spider silk proteins (~ 30%), therefore modules could be designed without the need to search for restriction endonuclease recognition sites which, after translation, match authentic amino acid sequences. Since the arrangement of the cloning cassette's elements remained unchanged upon cloning and multimerization, a variety of module combinations could be constructed (Fig.1D).

Design, synthesis and purification of synthetic spider silks. The inventors chose the dragline silk proteins ADF-3 and ADF-4 (3) from the garden spider *Araneus diadematus* as templates for the synthetic constructs. The partially identified primary structure of ADF-3 largely consists of repeat units, which all comprise a consensus sequence including a poly-alanine motif. Length of individual repeat units is determined by varying numbers of the motif GPGQQ. To mimic the repetitive sequence of ADF-3 we designed two modules. One module, termed A, was derived from the poly-alanine containing consensus sequence (Fig.1E). A second module termed Q contained four repeats of the GPGQQ motif. To study different length repeat units, one or two Q modules were combined with one A module to obtain (AQ) or (QAQ). These repeat units were multimerized to generate synthetic genes coding for the repetitive proteins (rep-proteins) (AQ)₁₂ and (QAQ)₈.

The repetitive part of ADF-4 is generally composed of a single conserved repeat unit displaying only slight variations. The inventors combined these variations and designed one consensus module termed C (Fig.1E), which the inventors multimerized to obtain the rep-protein C₁₆. The number of module repeats in all synthetic genes was chosen to code for proteins of similar molecular mass (~ 50 kDa).

ADF-3 and ADF-4 both display homologous NR-regions at their carboxyl termini, comprising 124 and 109 amino acids respectively. Gene sequences coding for these regions were amplified

by PCR, and codons problematic for bacterial expression were changed to more suitable codons by site directed mutagenesis (see materials and methods). Therefore, all of the synthetic genes used could be combined with the appropriate authentic NR-regions yielding genes coding for the repNR-proteins (AQ)₁₂NR3, (QAQ)₈NR3 and C₁₆NR4. Additionally NR3 and NR4 could be expressed alone.

After bacterial synthesis silk proteins were purified by a heat step followed by an ammonium sulfate precipitation. The identity of the proteins was confirmed by immunoblotting, using antibodies directed against T7 peptide tag sequences, attached to the amino-terminal end of all silk proteins (Fig.2A). Although all rep-proteins and all repNR-proteins had similar molecular weights (Table 1) they displayed different migration velocities when subjected to SDS-PAGE. This effect might be caused by aberrant binding of dodecylsulfate to the proteins due to different amino acid composition, leading to variation of the proteins' net charges. Besides full length proteins, immunoblotting revealed traces of proteins with lower molecular weight within preparations of repNR-proteins. Binding of the anti T7-tag antibody to these proteins identified them as silk proteins lacking part of their carboxyl-terminal end. Analyzing each purified protein by SDS-PAGE and silver staining, no further proteins were detected in all protein preparations (Fig.2B). Protein purity additionally was determined by measuring fluorescence emission. Incident light of 280 nm wavelength leads to excitation and fluorescence emission of tyrosines and tryptophanes while light of 295 nm exclusively excites the latter. Since none of the designed spider silk proteins comprised tryptophanes, fluorescence emission upon excitation with 295 nm would have been indicative for contaminating *E.coli* proteins, which on average contain 1.5% of tryptophane (30). Fluorescence measurements of all silk protein preparations revealed emission spectra akin to the spectrum of tyrosine, which occurs abundantly in the silk proteins. In contrast, no tryptophane fluorescence could be detected, indicating high purity of the protein preparations (data exemplarily shown for C₁₆NR4 in Fig.2B).

Bacterial production of synthetic silk proteins in Erlenmeyer flasks yielded similar protein amounts for all constructs. Yields of individual preparations ranged from 10 to 30 mg of purified protein per liter of culture medium. Fermentation of cells was employed in order to investigate

the possibility to up-scale protein synthesis. Yields of (QAQ)₈NR3 and C₁₆NR4 thus could be increased to 140 and 360 mg/l, respectively.

RepNR-proteins consist of a poorly structured repetitive region and a highly structured non-repetitive domain. Secondary structure was investigated by CD spectroscopy. Rep-proteins unveiled spectra typical for intrinsically unstructured proteins. In contrast NR-proteins revealed spectra indicative of high secondary structure content. These regions seem to represent independently folding protein domains. Spectra of repNR-proteins roughly corresponded to a combination of the rep- and NR-spectra weighted according to their share in the repNR-proteins. Although a minor structural change within the rep-regions or NR-domains upon mutual linking can not be excluded it is likely that the repNR-proteins are composed of a region displaying mostly random coil structure and a carboxyl terminal folded protein domain. Strikingly spectra of repNR-proteins were similar to CD-spectra obtained from the major ampullate silk dope directly extracted from spiders (*Nephila clavipes*) (9).

Silk proteins refold after thermal and chemical denaturation. Investigating structural changes by CD-spectroscopy upon heating, no cooperative temperature transitions were observed for rep-proteins between 20°C and 90°C, an effect which has also been observed for other intrinsically unfolded proteins (31;32) (Fig.3). Since repNR-proteins were at least partially structured, thermal unfolding of the structured region should be detectable at elevated temperatures. Accordingly, cooperative thermal transitions were observed. Midpoints of temperature transitions were 67°C ((QAQ)₈NR3), 66°C ((AQ)₁₂NR3) and 72°C (C₁₆NR4), respectively (Fig.3B and Table 1). Furthermore, all thermal transitions were completely reversible. The reversibility of the structural changes upon heating explained the high recovery of soluble silk proteins after the heat step employed during protein purification. Tris was used to buffer all solutions investigated by CD spectroscopy, because of good spectral properties and little capacity to promote silk protein aggregation. Due to the strong temperature dependence of Tris buffered solutions, the pH of the samples was expected to shift from pH 8 to pH 6 upon heating from 20°C to 90°C (19). However, temperature transitions of silk proteins in phosphate buffer at pH 8, displaying a temperature independent pK-value, revealed equal midpoint temperatures (data not shown) although they were not entirely reversible probably due to protein aggregation

(see below). This indicated that thermal transitions of silk proteins were not influenced by thermally induced changes of the pH in Tris-buffered solutions.

The effect of chemical de- and renaturation on secondary structure was investigated by measuring circular dichroism of repNR-proteins in Tris buffer, after dialysis against 6 M GuaHCl and renaturation by dialysis against Tris Buffer. The identical spectra of the initial and the refolded proteins indicated that chemical denaturation is reversible (data not shown).

The solubility of silk proteins is determined by their repetitive sequences. In order to gain high protein concentrations in the dope, silk proteins have to be highly soluble. We tested the maximum concentrations at which rep- and repNR-proteins remained soluble to identify primary structure elements determining solubility. All proteins comprising the modules A and Q could be concentrated by ultra filtration to more than 30% w/v without forming visible aggregates, regardless of the presence of the NR-domain. In contrast, proteins containing the module C could only be concentrated to 8% w/v (C₁₆) and 9% w/v (C₁₆NR4), respectively (Table 1). Both proteins formed a gel-like solid upon further concentration (data not shown). Thus, solubility of the silk proteins was solely determined by their repetitive sequences and was not influenced by the NR-domain.

Potassium does not promote aggregation of synthetic silk proteins, independent of their primary structure. pH, ions, such as potassium and phosphate, and mechanical stress are involved in natural silk assembly. Here we wanted to investigate how these factors promote the assembly of synthetic silk proteins. Since we were unable to imitate the authentic assembly process, which requires pre-orientation of the involved proteins as found in the liquid crystalline dope (33), we performed an aggregation assay starting with protein solutions not displaying orientational order. None of the tested rep-, repNR- and NR-proteins displayed significant aggregation (< 5%) when incubated in buffer, indicating that all proteins were intrinsically soluble under the testing conditions (Fig.4). To investigate whether addition of ions caused aggregation by increasing the ionic strength, proteins were incubated with sodium chloride. However no aggregation was observed. In contrast to sodium, potassium has previously been reported to specifically promote

silk aggregation (34). Yet, potassium chloride also showed no influence on solubility of the synthetic silk proteins (Fig.4).

Acidification and addition of phosphate initiate aggregation of rep-proteins depending on their primary structure. The exact function of acidification during spider silk assembly has not yet been determined. However it seems likely that negatively charged groups (e.g. phosphoryl groups) are protonated thus reducing the net charge and repulsion of spider silk proteins. Since the synthetic silk proteins did not contain chemical groups displaying a pK_A -value within the range of the pH-shift observed during the spinning process, the inventors aimed to mimic this effect by protonating all terminal and side chain carboxyl-groups. (QAQ)₈ and (AQ)₁₂, displaying only the terminal carboxyl group, showed no (<5%) and weak (18%) aggregation at pH 1. Interestingly protonation C₁₆'s 16 glutamate residues also caused only weak aggregation (8%) (Fig.4). Phosphate which has been described to be added to the dope during the spinning process caused no aggregation of (QAQ)₈ and weak precipitation of C₁₆ (12%). In contrast, (AQ)₁₂ displayed an increased tendency to aggregate (47%) after treatment with potassium phosphate. Similar results were obtained using sodium phosphate, indicating that the effect is specifically caused by phosphate ions (data not shown).

NR-domains amplify the response to factors that promote aggregation. To investigate the influence of NR-domains, aggregation of repNR-proteins as well as NR-proteins at low pH and upon treatment with phosphate was tested. Acidification of (QAQ)₈NR3 and (AQ)₁₂NR3, as well as NR3 caused weak aggregation (10%, 15% and 13%), which was in the range displayed by the corresponding rep-proteins. Interestingly, although the NR4-domain did not precipitate at pH 1 (0%), C₁₆NR4 showed strong aggregation at pH 1 (70%). Thus the combination of the repetitive C₁₆ and the NR4-domain, which did not significantly aggregate upon acidification, led to a protein highly sensitive to this aggregation promoting factor. Similar results were obtained for the addition of phosphate. While neither NR3 nor NR4 showed aggregation in the presence of phosphate (1% and 0%), the addition of the NR-domains to the repetitive regions caused an increased aggregation of the repNR-proteins in comparison to rep-proteins ((QAQ)₈NR3: 57%, (AQ)₁₂NR3: 81%, C₁₆NR4: 80%).

Using a cloning strategy that allows seamless and controlled assembly of DNA modules, synthetic genes were constructed coding for spider silk-like proteins. The design of proteins yielded different combinations of repeat units and naturally occurring NR-regions, to systematically test the properties of such single primary structure elements. Structural analysis by CD-spectroscopy revealed that repetitive regions are mostly unstructured in their soluble state, displaying properties common to other intrinsically unfolded proteins (31;32). The same conformational state as been proposed for the largest part of the major ampullate content (10) which is dominated by repetitive protein sequences. In contrast NR-regions were found to represent independently folding protein domains that adopt their conformation after heat denaturation as well as treatment with chaotropic agents. Because of their relative small size compared to repetitive regions the influence on overall structural properties was small in repNR-proteins.

In natural spider silks displaying repetitive regions of several hundred kDa the structural contribution of the NR-regions can be expected to be even smaller, explaining the missing evidence for their presence in investigations of major ampullate content. Because of the reversibility of thermal and chemical denaturation of repNR-proteins and the similarity of CD data presented in this work and obtained from natural silk dope, it can be assumed that even after treatment with heat and chaotropic reagents during purification and sample preparation all investigated spider silk components in aqueous solutions were in a conformational state comparable to that of natural silk proteins within the dope.

According to Uversky et al. intrinsic unfolding of proteins can be predicted based on their net charge and mean hydropathicity. The net charge of a protein is used to calculate a "boundary" hydropathicity. If the mean hydropathicity of the protein is below the "boundary" value, the protein is predicted to be intrinsically unfolded (35;36). In accordance with the presented results the repetitive sequences (QAQ)₈ and (AQ)₁₂ are predicted to be intrinsically unfolded (Table 1). Intrinsic unfolding of a protein means that interactions of the amino acid residues with the surrounding solvent are more favourable than with amino acids of the same or other polypeptide chains. Accordingly, (QAQ)₈ and (AQ)₁₂ are soluble even at high concentrations. In contrast, C₁₆ displays a hydropathicity slightly above the boundary value. While still revealing properties of

intrinsically unfolded proteins interactions between polypeptide chains are becoming more favorable at high concentrations leading to aggregation of the protein and resulting in a lower solubility compared with (QAQ)₈ and (AQ)₁₂ (Table 1).

As repetitive sequences constitute the largest fraction of spider silk proteins, they likely determine many of the proteins' properties. Accordingly solubilities of repNR-proteins do not significantly differ from rep-proteins. The solubility and calculated hydropathicity of (QAQ)₈ and (AQ)₁₂ correlate well with the values of authentic ADF-3 (Table 1). C₁₆ and ADF-4 both display lower solubility, although C₁₆ does not share the high intrinsic insolubility of ADF-4. This difference can be explained by higher hydropathicity and lower net charge of ADF-4 compared to C₁₆.

In contrast to repetitive regions, NR-domains represent only a small fraction of spider silk proteins. Both NR-domains revealed a structure rich in α -helices. Due to the high similarity between the NR-domains of ADF-3 and ADF-4 (81% similarity and 67% identity) it can be assumed, that both might fulfill related functions. Further information about the function of the NR-domains was obtained when investigating aggregation of the silk proteins upon treatment with factors known to induce the assembly of silk proteins *in vivo*. Reducing negative charges by protonation of the silk proteins' carboxyl groups was expected to mainly affect proteins comprising the C module. Accordingly, proteins composed of modules A and Q, which do not contain aspartates or glutamates, did not show more than weak aggregation. C₁₆ even after neutralization of its 16 negative charges remained mostly soluble. Strikingly the combination of the NR4-domain, which did not show any response to acidification by itself, and the weakly aggregating C₁₆ resulted in a protein highly sensitive to protonation. Thus charge reduction of the repetitive region and the presence of the NR-domain are required for efficient aggregation. Similar results were obtained when phosphate was added to the protein solutions. Phosphate, like other lyotropic ions is known to increase the surface tension of water, promoting hydrophobic interactions (37). In the case of spider silk proteins it is likely that the addition of phosphate initiates interactions between the hydrophobic poly-alanine motifs, causing the aggregation of the proteins. Accordingly aggregation of (AQ)₁₂ was higher than of (QAQ)₈ which contains one third less poly-alanine motifs than (AQ)₁₂. C₁₆ displaying the longest and highest number of

poly-alanine motifs however did not show the strongest aggregation upon phosphate treatment. A possible explanation for this unexpected result might be the repulsion of the negatively charged glutamate side chains and phosphate ions leading to their exclusion from the surrounding solvent and a weakening of their lyotropic effect. Even though both NR-domains did not respond to the addition of phosphate, their addition to the rep-proteins strongly increased phosphate sensitivity. Although the presented data is not sufficient to draw a final conclusion it seems likely that the NR-domains function as unspecific enhancers of sensitivity to aggregation promoting factors. For efficient aggregation their presence is as important as the ability of repetitive regions to respond to these factors.

The mechanism of this enhancement might involve changes in the oligomeric status of the silk proteins. NR-domains have been found to form disulfide bridged dimers (38). Further oligomerization might lead to increased local concentrations of polypeptide sequences required for initiating aggregation which is assisted by solvent conditions that favour the formation of intermolecular interactions.

The present protein engineering approach, which combines synthetic repetitive sequences with authentic NR-regions, reveals that proteins closely resembling authentic silk proteins can be produced at high yields. The bacterial expression system as well as the simple and cheap purification process, which can easily be scaled up, provides the basis for cost-efficient industrial scale production of spider silk-like proteins. Based on the present studies, the molecular mechanisms of spider silk assembly can be further investigated, which will provide the knowledge required for artificially spinning silk threads from recombinant proteins and for gaining new materials for biotechnological and medical applications.

Assembly of spider silk derived proteins

The following experiments were performed to demonstrate that proteins derived from spider silk sequences ADF-3 (SEQ ID NO:1) or ADF-4 (SEQ ID NO:2) can be assembled into morphological distinct forms. Proteins (AQ)₂₄NR3 and C₁₆NR4 were constructed, produced and prepared in aqueous solutions as described in *Biochemistry* 2004 Vol.43 pp. 13604-11362. If not

otherwise mentioned protein solutions contained 10 mM Tris-(hydroxymethyl)-aminomethan (Tris) pH 8.0.

1. Spheres

Protein spheres displaying diameters ranging between 0.5 and 2 μm (Fig. 7a) were generated by adding 0.8 M ammonium sulphate to a 0.2% (w/v) C_{16} solution.

2. Nanofibrils

Nanofibrils displaying diameters between 0.7 and 4 nm (Fig. 7b) were formed by incubating a 1% (w/v) $\text{C}_{16}\text{NR4}$ solution at room temperature for 2 weeks.

3. Microfibrils

For the formation of microfibrils 5 - 10 μl of a 25% (w/v) $(\text{AQ})_{24}\text{NR3}$ solution were slowly injected into 0.5 M potassium phosphate pH 8.0, forming a stable drop of protein solution. After incubation for 1 min the protein drop was removed from the solution using tweezers. After an additional incubation time of 1 min in air a protein fibril could be drawn from the protein drop at a rate of approximately 2 cm/s using a second set of tweezers. The fibrils displayed a round cross section with a diameter of 4 μm (Fig 7c,d).

4. Foams

Protein foams (Fig. 7e,f) were generated from solutions containing 2.5 mM ammonium peroxodisulfate (APS), 100 μM tris(2,2'-bipyridyl)dichlororuthenium(II) (Rubpy) and 10% (w/v) $(\text{AQ})_{24}\text{NR3}$ or 2% (w/v) $\text{C}_{16}\text{NR4}$. The protein solutions were frothed up with air. To stabilize the resulting foam structure proteins were crosslinked by exposition to visible light from a tungsten lamp for 1 min (Protocol: *PNAS* 1999 Vol.96 pp.6020-6024). Foams were subsequently dried at 95°C.

5. Gels

$\text{C}_{16}\text{NR4}$ nanofibrils at 1% (w/v) concentration displayed a gel like appearance which easily could be disrupted by agitation or shearing. To improve the mechanical properties of the gel APS and Rubpy were allowed to enter the gel by diffusion to yield final concentrations of 10 mM APS

and 100 μ M Rubpy. After light induced crosslinking (see section 4) dimensionally stable gels could be obtained (Fig. 7g).

6. Films

6.1 Soluble state of spider silk proteins

In order to cast films the inventors used the two synthetic silk proteins, (AQ)₂₄NR3 and C₁₆, which are derived from the dragline silk proteins ADF-3 and ADF-4 from the garden spider *Araneus diadematus* (see also above for further explanations). They chose these two different proteins based on previous observations that ADF-3 and ADF-4 as well as its derivatives display a markedly different behaviour regarding solubility and assembly. Aqueous solutions of both proteins could be prepared by dissolving lyophilized proteins in 6 M guanidinium thiocyanate and subsequent removal of the salt by dialysis against a low salt buffer such as 5 mM potassium phosphate pH 8.0. Lyophilized proteins could also be directly dissolved in HFIP. Measuring circular dichroism (CD) of protein solutions revealed a different influence of the two solvents on secondary structure. In aqueous solution both proteins displayed a CD-spectrum with a single minimum at a wavelength below 200 nm which is indicative of a mainly random coiled protein (Fig.8). In contrast, the spectra of both proteins in HFIP displayed one minimum at 201 – 202 nm and an additional minimum ((AQ)₂₄NR3) or shoulder (C₁₆) at 220 nm which is indicative of an increased α -helical content (Fig.8).

6.2 Film formation

Films were cast on a polystyrene surface (or on quartz glass for CD-measurements) from HFIP solutions containing 2% w/v protein. After evaporation of the solvent, (AQ)₂₄NR3 and C₁₆ both formed transparent films that could easily be peeled off the surface (Fig.9 and data not shown). Assuming complete evaporation of the solvent and the density of the protein film to be identical with the reported value of 1.3 g/cm³ for spider dragline silk, the thickness of the films was calculated to range from 0.5 to 1.5 μ m. As-cast (freshly prepared) films made of either protein dissolved upon contact with water. Since water insolubility is a prerequisite for most applications of protein films, the inventors looked for a processing method in order to render films insoluble. Potassium phosphate has been known to induce aggregation and formation of chemically stable

structures of the employed silk proteins. Accordingly, processing (incubating) of as-cast films with 1 M potassium phosphate resulted in the conversion of films into a water insoluble state.

6.3 Secondary structure

To investigate the structural properties of the protein films, their secondary structure was investigated by CD-spectroscopy. As-cast films revealed a spectrum with two minima at 208 nm and 220 nm, indicative of a high α -helical content (Fig.10). After processing with 1 M potassium phosphate, films revealed spectra with a single minimum at 218 nm which is typical for a β -sheet rich structure. Thus, the transition from water solubility to water insolubility paralleled a conversion of the protein's secondary structure from α -helix to β -sheet.

6.4 Chemical stability

To test the chemical stability, films were exposed to 8 M urea, 6 M guanidinium hydrochloride and 6 M guanidinium thiocyanate (Table 2). As-cast films of both proteins as well as processed films of (AQ)₂₄NR3 were soluble in these denaturants. In contrast, processed films of C₁₆ could only be dissolved in guanidinium thiocyanate. This remarkable chemical stability of C₁₆ films is identical to that of recombinantly produced and assembled ADF-4 and that of natural dragline silk. Previous studies correlated assembly properties and stabilities of assembled structures directly with the amino acid sequences of the silk proteins. It therefore can be concluded, that properties of spider silk films can directly be modified by altering the primary structure of the silk protein via manipulation of the corresponding silk gene.

6.5 Film modification

Many applications of protein films require the presence of specific functionalities on the film's surface. In order to demonstrate, that our spider silk protein films can be modified with small organic molecules as well as biological macromolecules like proteins, the chromophor fluorescein and the enzyme β -galactosidase were chemically coupled to processed C₁₆ films. The coupling was achieved by activating surface exposed carboxyl groups of C₁₆ using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (for details of the reactions see supplementary material indicated below). The films were then incubated with ethylenediamine leading to the formation of an amide. The remaining free amino group of ethylenediamine was subsequently

coupled to fluorescein isothiocyanate resulting in the efficient covalent linkage of the fluorescein (Fig.11A) via formation of a stable thiourea derivative. Similarly, incubation of β -galactosidase with EDC-activated C_{16} films led to the formation of amide bonds between carboxyl groups of C_{16} and primary amines (e.g. from lysine residues) of β -galactosidase which were accessible at the enzyme's surface. After repeated washing of such modified films, β -galactosidase activity could be detected using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as a substrate (Fig.11B).

6.6 Conclusion

Herein, it could be demonstrated that protein films can be obtained from synthetic spider silk proteins. The films, which initially were water soluble, can be processed with potassium phosphate leading to water-insolubility which is a major requirement for many applications. Comparison of the chemical stabilities of films made from two different synthetic spider silk proteins suggests that the properties of the films are based on the primary structure of the proteins. Thus, it will be possible to generate silk proteins that form films displaying specific properties. Since different functional molecules can be covalently attached to the film's surface, a great variety of technical or medical applications can be approached in the future.

6.7 Supplementary materials and results

Preparation of protein solutions

Protein production and purification was performed as described previously. To obtain aqueous solutions of $(AQ)_{24}NR3$ and C_{16} , lyophilized protein was dissolved in 6 M guanidinium thiocyanate at a concentration of 10 mg/ml and subsequently dialyzed against 5 mM potassium phosphate pH 8.0. Aggregates were removed by sedimentation at $15,000\times g$ for 10 min. Protein concentrations were determined photometrically in a 1cm path length cuvette at 276 nm using calculated extinction coefficients of $73950\text{ M}^{-1}\text{cm}^{-1}$ for $(AQ)_{24}NR3$ and $46400\text{ M}^{-1}\text{cm}^{-1}$ for C_{16} . Alternatively, lyophilized silk proteins were dissolved directly in hexafluoroisopropanol (HFIP).

Secondary structure analysis

Far-UV circular dichroism (CD) spectra were obtained using a Jasco 715 spectropolarimeter (Jasco International Co. Ltd., Tokyo, Japan). Spectra of soluble proteins were taken at a protein concentration of 200 $\mu\text{g/ml}$ in 5 mM potassium phosphate (pH 8.0) or HFIP in a 0.1 cm path length quartz cuvette at 20 °C. For measuring films, 100 μl of a 2 mg/ml protein solution in HFIP were spread on a plain quartz glass of 4 cm^2 and air-dried before CD-measurement. Scan speed was 20 nm/min, step size was 0.2 nm, integration time was set to 1 s and band width was 1 nm. Four scans were averaged.

Film modification

1. Coupling of fluorescein to C_{16} film surfaces

Films were prepared by spreading 15 μl per well of 20 mg/ml C_{16} in HFIP on the bottom of a 24-well plate. After evaporation of HFIP, films were incubated for 5 minutes with 1 M potassium phosphate. After rinsing with water, carboxyl groups were activated by incubation for 15 min with 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.0, 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 20 mM N-hydroxysulfo-succinimide (NHS). Subsequently ethylenediamine was added to yield a final concentration of 500 mM. After 2 h of incubation films were thoroughly rinsed with water. Finally, films were incubated for 1 h with 1 mg/ml fluorescein isothiocyanate in 100 mM sodium carbonate pH 9.0, followed by rinsing with water and air-drying.

2. Coupling of β -galactosidase to C_{16} film surfaces

Films were prepared and activated as described above. After 15 min of incubation with EDC / NHS, films were rinsed with water and subsequently incubated for 2 h with a solution containing 100 $\mu\text{g/ml}$ β -galactosidase, 4 mM KH_2PO_4 , 16 mM Na_2HPO_4 , 115 mM NaCl (PBS). After thorough rinsing with PBS, enzymatic activity was tested on the film surface.

β -galactosidase assay

β -galactosidase coupled films were incubated for 16 h at room temperature with a solution containing 100 mM sodium phosphate pH 7.0, 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM β -mercaptoethanol and 2 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal).

7. Additional hydrogels

The repetitive part of ADF-4 is generally composed of a single conserved repeat unit displaying only slight variations. The inventors combined these variations and designed one consensus module termed C (GSSAAAAAASGPGGYGPENQGPGSGPGGYGPGGP) (SEQ ID NO: 5), which was multimerized to obtain the rep-protein C₁₆, which will result in a protein of a molecular mass of 48 kDa.

C₁₆ silk gene was expressed in the *E. coli* strain BLR [DE3] (Novagen). Cells were grown at 37°C in LB medium to an OD₆₀₀ = 0.5. Before induction with 1 mM IPTG (Isopropyl-β-D-thiogalactosid), cells were shifted to 25°C. Cells were harvested after 3-4 hours of induction.

C₁₆ protein was purified as described in Huemmerich *et al* (40). Pellets of C₁₆ were washed with 8 M urea and dissolved in 6 M guanidinium thiocyanate (GdmSCN) before dialysis against 10 mM NH₄HCO₃. Precipitates formed during dialysis were removed by sedimentation at 50,000×g for 30 min and the remaining soluble silk proteins were lyophilized. Prior to analysis lyophilized protein was dissolved in 6 M GdmSCN followed by dialysis against 10mM Tris / HCl. Aggregates were removed by sedimentation at 125,000×g for 30 min. Protein concentrations were determined photometrically in a 1cm path length cuvette at 276 nm using calculated extinction coefficient (40).

C₁₆ self-assembled into nanofibers at concentrations between 5 and 30 mg/ml after the addition of 10 % w/v of methanol (Fig. 12). Strikingly, at the concentrations used the nanofibers led to the formation of fiber network representing hydrogels. C₁₆ hydrogels could easily be disrupted by agitation or shearing. To improve the mechanical properties of the gel ammonium peroxodisulfate (APS), and Tris(2,2'-bipyridyl)dichlororuthenium(II) (Rubpy) were allowed to enter the gel by diffusion to yield final concentrations of 10 mM APS and 100μM Rubpy. To gain dimensionally stable gels proteins were crosslinked by exposition to visible light from a tungsten lamp for 1 min (IV) (Fig. 13).

Dynamic rheological measurements of the crosslinked and non-crosslinked hydrogels were performed using a Physica MCR 301 with a 25 mm Plate-Plate geometry. The gap between the upper plate and the sample dish was set by first moving the upper plate approximately 2 mm above the surface of the sample. The upper plate was lowered very slowly (5 $\mu\text{m/s}$), while monitoring the normal force and was stopped at a limit normal force of 0.1 N.

After finding adequate gap sizes for the samples, the samples were sheered at 0.5 Hz and 1% deformation until the normal force equilibrated to a constant value. The dynamic rheology measurements were performed at room temperature by applying a constant stress to the sample. Rheological measurements were conducted on samples with protein concentrations ranging from 5 to 30 mg/ml.

AFM images of the dried hydrogels indicate that the nanofibers are approximately 3 nm in diameter and appear to be semiflexible, with a persistence length on the same order of magnitude as their length (Figure 12). Many of the nanofibers also appear to have a branched structure. From the AFM images it could not be determined, if the branch-like structures are physical branches in each polymer fiber or are a result of nanofiber bundling.

Similar to most concentrated polymer networks the hydrogel of recombinant C₁₆ spider silk protein demonstrates viscoelastic behavior. When a stress is applied to the viscoelastic C₁₆ silk networks the strain changes slowly with time and is proportional to the applied stress. Figure 14 shows the stress/strain behavior of the crosslinked and non-crosslinked hydrogels at a concentration of 10 mg/ml. The non-crosslinked C₁₆ silk hydrogel has an initial shear modulus of 38 Pa. However, as stress is increased the non-crosslinked hydrogel shows a higher deformation response to stress, and after a strain of 20% the response is relatively linear. With increasing stress the network continues to deform until a strain of 90% is reached, where the non-crosslinked hydrogel ruptures and flows. Unlike the non-crosslinked fiber networks, the crosslinked networks show a linear viscoelastic response over all strains, has a much higher shear modulus of 820 Pa, and ruptures at a lower strain of 30%.

Dynamic viscoelastic measurements of the non-crosslinked fiber networks at a polymer concentration of 20 mg/ml reveal that the storage modulus (G') and the loss modulus (G'') are very dependent on the oscillation frequency (ω) in both the high ω and low ω range (Figure 15). The network demonstrates viscous behavior at low frequencies and elastic behavior at moderate frequencies with a crossover at 0.49 Hz. The observed behavior of the hydrogel is similar to that expected for an entangled polymer network and not similar to what would be expected from a liquid crystalline solution or viscous fluid.

The non-crosslinked C_{16} silk hydrogel also displays dynamic viscoelastic behavior that is much different than that which is observed in the chemically crosslinked hydrogels (Figure 15). Unlike the behavior of the non-crosslinked fiber network, the storage modulus of the crosslinked fiber network is nearly constant at all frequencies, except at the highest frequencies tested. The crosslinked C_{16} silk hydrogel also demonstrates a higher storage and lower loss modulus than that which is observed in the non-crosslinked network.

As would be expected, the storage modulus of the crosslinked hydrogel is higher than that of the non-crosslinked network for all concentrations tested (Figure 16). However, unexpectedly the storage moduli of both crosslinked and non-crosslinked networks increase with concentration $[c]$ and have a $[c]^2$ dependence. In the case of crosslinked linear semiflexible biopolymer networks, where the persistence length is larger than the mesh size, the storage modulus of the polymer network is expected to have a dependence of $[c]$, which is close to that of the crosslinked C_{16} silk hydrogel. In the case of linear semiflexible biopolymer networks that are entangled but not crosslinked, the storage modulus is expected to have a much lower concentration dependence of $[c]$. Such a dependency has been shown to be valid for other biopolymers such as F-actin, but does not describe the dependency of the non-crosslinked silk hydrogel.

This discrepancy could be explained if the branch-like structures observed in the AFM images are real physical branches in the polymer network. The storage modulus of a branched semiflexible polymer network would be expected to show a concentration dependency between what would be expected for the crosslinked and non-crosslinked polymer network.

The AFM images and rheology data is consistent with from the model of a branched semiflexible polymer network. However, the storage modulus scaling behavior of the hydrogels can not be explained within the framework of the most widely excepted models for linear semiflexible polymer networks.

TABLE 1

Selected properties of synthetic silk constructs and authentic spider silk proteins ADF-3 and ADF-4.

	QAQ ₈	AQ ₁₂	C ₁₆	NR3	NR4	QAQ ₈ N R3	AQ ₁₂ N R3	C ₁₆ NR 4	ADF- 3	ADF- 4
molecular mass [kDa] ^a	47.5	48.1	47.7	13.3	11.9	59.3	59.8	58.1	56.1	34.9
extinction coefficient (276nm) [M ⁻¹ cm ⁻¹] ^b	23200	34800	46400	4423	1523	27550	39150	47850	n.d.	n.d.
charged amino acid residues ^c (positive / negative)	0 / 0	0 / 0	0 / 16	2 / 2	2 / 2	2 / 2	2 / 2	2 / 18	4 / 2	2 / 6
grand average of hydropathicity (GRAVY) ^d	-1.252	-0.987	-0.464	0.401	0.438	-0.918	-0.710	-0.294	-0.628	-0.075
normalized hydropathicity and "boundary" mean hydropathicity ^e	0.361 0.413	0.390 0.413	0.448 0.440	0.545 0.413	0.548 0.413	n.d. n.d.	n.d. n.d.	n.d. n.d.	0.399 0.415	0.464 0.417
midpoint temperature of thermal unfolding ^f	no	no	no	n.d.	n.d.	67°C	66°C	72°C	n.d.	n.d.
solubility (w/v) ^g	> 30%	> 30%	8%	n.d.	n.d.	> 30%	> 30%	7%	> 28%	< 1%

^a Molecular mass of engineered proteins includes the T7-tag.

^b Extinction coefficients were calculated according to Gill & Hippel (23).

^c Charged amino acid residues refer to silk gene sequences only; T7-tags comprise an additional arginine.

^d Hydropathicity was calculated as described previously (39). Hydrophobicity increases with hydropathicity values.

^e Hydropathicity was normalized to a range between 0 and 1. "Boundary" hydropathicity was calculated according to Uversky et al. (35;36). If normalized hydropathicity values are below the "boundary" value proteins are predicted to be intrinsically unfolded. Values of ADF-3 and ADF-4 refer to their repetitive sequences only.

^f Midpoint temperatures were determined by CD spectroscopy.

^g Values for ADF-3 and ADF-4 were taken from (18) and unpublished results.

Table 2 Solubility of protein films in denaturants. Films were regarded to be insoluble (-), in case complete immersion in the respective agent and repeated shaking over a period of five minutes did not result in a change of optical appearance. In contrast, solubility (+) was marked by the complete disintegration of the film under the same conditions.

	water	8 M urea	6 M guanidinium hydrochloride	6 M guanidinium thiocyanate
(AQ) ₂₄ NR3 as-cast	+	+	+	+
(AQ) ₂₄ NR3 processed	-	+	+	+
C ₁₆ as-cast	+	+	+	+
C ₁₆ processed	-	-	-	+

REFERENCES

1. Gosline, J. M., Guerette, P. A., Ortlepp, C. S., and Savage, K. N. (1999) The mechanical design of spider silks: from fibroin sequence to mechanical function, *J. Exp. Biol.* 202 Pt 23, 3295-3303.
2. Vollrath, F. and Knight, D. P. (2001) Liquid crystalline spinning of spider silk, *Nature* 410, 541-548.
3. Guerette, P. A., Ginzinger, D. G., Weber, B. H., and Gosline, J. M. (1996) Silk properties determined by gland-specific expression of a spider fibroin gene family, *Science* 272, 112-115.
4. Gatesy, J., Hayashi, C., Motriuk, D., Woods, J., and Lewis, R. (2001) Extreme diversity, conservation, and convergence of spider silk fibroin sequences, *Science* 291, 2603-2605.
5. Simmons, A. H., Ray, E., and Jelinski, L. W. (1994) Solid-State ^{13}C NMR of *Nephila clavipes* Dragline Silk Establishes Structure and Identity of Crystalline Regions, *Macromolecules* 27, 5235-5237.
6. Parkhe, A. D., Seeley, S. K., Gardner, K., Thompson, L., and Lewis, R. V. (1997) Structural studies of spider silk proteins in the fiber, *J. Mol. Recognit.* 10, 1-6.
7. van Beek, J. D., Hess, S., Vollrath, F., and Meier, B. H. (2002) The molecular structure of spider dragline silk: folding and orientation of the protein backbone, *Proc. Natl. Acad. Sci. U. S. A* 99, 10266-10271.
8. Hijirida, D. H., Do, K. G., Michal, C., Wong, S., Zax, D., and Jelinski, L. W. (1996) ^{13}C NMR of *Nephila clavipes* major ampullate silk gland, *Biophys. J.* 71, 3442-3447.
9. Kenney, J. M., Knight, D., Wise, M. J., and Vollrath, F. (2002) Amyloidogenic nature of spider silk, *Eur. J. Biochem.* 269, 4159-4163.
10. Hronska, M., van Beek, J. D., Williamson, P. T., Vollrath, F., and Meier, B. H. (2004) NMR characterization of native liquid spider dragline silk from *Nephila edulis*, *Biomacromolecules* 5, 834-839.
11. Kerkam, K., Viney, C., Kaplan, D., and Lombardi, S. (1991) Liquid crystallinity of natural silk secretions, *Nature* 349, 596-598.
12. Knight, D. P. and Vollrath, F. (1999) Liquid crystals and flow elongation in a spider's silk production line, *Proc. R. Soc. Lond.* 519-523.

13. Willcox, J., Gido, S., Muller, W., and Kaplan, D. (1996) Evidence of a Cholesteric Liquid Crystalline Phase in Natural Silk Spinning Processes, *Macromolecules* 29, 5106-5110.
14. Knight, D. P. and Vollrath, F. (2001) Changes in element composition along the spinning duct in a Nephila spider, *Naturwissenschaften* 88, 179-182.
15. Vollrath, F., Knight, D., and Hu, X. W. (1998) Silk production in a spider involves acid bath treatment, *Proc. R. Soc. Lond B Biol. Sci.* 265, 817-820.
16. Tillinghast, E. K., Chase, S. F., and Townley, M. A. (1984) Water extraction by the major ampullate duct during silk formation in the spider, *Argiope aurantia* Lucas, *J. Insect Physiol.* 30, 591-596.
17. Knight, D. P., Knight, M. M., and Vollrath, F. (2000) Beta transition and stress-induced phase separation in the spinning of spider dragline silk, *Int. J. Biol. Macromol.* 27, 205-210.
18. Lazaris, A., Arcidiacono, S., Huang, Y., Zhou, J. F., Duguay, F., Chretien, N., Welsh, E. A., Soares, J. W., and Karatzas, C. N. (2002) Spider silk fibers spun from soluble recombinant silk produced in mammalian cells, *Science* 295, 472-476.
19. Sambrook, J. and Russell, D. (2001) *Molecular Cloning*.
20. Kroll, D. J., Abdel-Malek Abdel-Hafiz, H., Marcell, T., Simpson, S., Chen, C. Y., Gutierrez-Hartmann, A., Lustbader, J. W., and Hoeffler, J. P. (1993) A multifunctional prokaryotic protein expression system: overproduction, affinity purification, and selective detection, *DNA Cell Biol.* 12, 441-453.
21. Reiling, H. E., Laurila, H., and Fiechter, A. (1985) Mass-Culture of Escherichia-Coli - Medium Development for Low and High-Density Cultivation of Escherichia Coli-B/R in Minimal and Complex Media, *Journal of Biotechnology* 2, 191-206.
22. Yee, L. and Blanch, H. W. (1992) Recombinant protein expression in high cell density fed-batch cultures of Escherichia coli, *Biotechnology (N. Y.)* 10, 1550-1556.
23. Gill, S. C. and von Hippel, P. H. (1989) Calculation of Protein Extinction Coefficients from Amino-Acid Sequence Data, *Analytical Biochemistry* 182, 319-326.
24. Arcidiacono, S., Mello, C., Kaplan, D., Cheley, S., and Bayley, H. (1998) Purification and characterization of recombinant spider silk expressed in Escherichia coli, *Appl. Microbiol. Biotechnol.* 49, 31-38.

25. Prince, J. T., McGrath, K. P., DiGirolamo, C. M., and Kaplan, D. L. (1995) Construction, cloning, and expression of synthetic genes encoding spider dragline silk, *Biochemistry* 34, 10879-10885.
26. Fahnestock, S. R. and Irwin, S. L. (1997) Synthetic spider dragline silk proteins and their production in *Escherichia coli*, *Appl. Microbiol. Biotechnol.* 47, 23-32.
27. Lewis, R. V., Hinman, M., Kothakota, S., and Fournier, M. J. (1996) Expression and purification of a spider silk protein: a new strategy for producing repetitive proteins, *Protein Expr. Purif.* 7, 400-406.
28. Scheller, J., Guhrs, K. H., Grosse, F., and Conrad, U. (2001) Production of spider silk proteins in tobacco and potato, *Nat. Biotechnol.* 19, 573-577.
29. Padgett, K. A. and Sorge, J. A. (1996) Creating seamless junctions independent of restriction sites in PCR cloning, *Gene* 168, 31-35.
30. Blattner, F. R., Plunkett, G., III, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12, *Science* 277, 1453-1474.
31. Kim, T. D., Ryu, H. J., Cho, H. I., Yang, C. H., and Kim, J. (2000) Thermal behavior of proteins: heat-resistant proteins and their heat-induced secondary structural changes, *Biochemistry* 39, 14839-14846.
32. Uversky, V. N., Lee, H. J., Li, J., Fink, A. L., and Lee, S. J. (2001) Stabilization of partially folded conformation during alpha-synuclein oligomerization in both purified and cytosolic preparations, *J. Biol. Chem.* 276, 43495-43498.
33. Knight, D. P. and Vollrath, F. (2002) Biological liquid crystal elastomers, *Philos. Trans. R. Soc. Lond B Biol. Sci.* 357, 155-163.
34. Chen, X., Knight, D. P., and Vollrath, F. (2002) Rheological characterization of nephila spidroin solution, *Biomacromolecules.* 3, 644-648.
35. Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) Why are "natively unfolded" proteins unstructured under physiologic conditions?, *Proteins* 41, 415-427.
36. Uversky, V. N. (2002) Natively unfolded proteins: a point where biology waits for physics, *Protein Sci.* 11, 739-756.

37. Arakawa, T. and Timasheff, S. N. (1985) Theory of protein solubility, *Methods Enzymol.* **114**, 49-77.
38. Sponner, A., Unger, E., Grosse, F., and Weisshart, K. (2004) Conserved C-termini of Spidroins are secreted by the major ampullate glands and retained in the silk thread, *Biomacromolecules.* **5**, 840-845.
39. Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein, *J. Mol. Biol.* **157**, 105-132.
40. Huemmerich, D., Helsen, C.W., Oschmann, J., Rudolph, R. and Scheibel, T. (2004) Primary structure elements of dragline silks and their contribution to protein solubility and assembly, *Biochemistry* **43**, 13604-13612.

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Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Gly Gly Tyr Gly Pro Gly

165	170	175
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Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Gly Gly Tyr Gly Pro		
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Gly Ser Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly		
210	215	220
Pro Gly Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala		
225	230	235
Ala Ala Gly Gly Tyr Gly Pro Gly Tyr Gly Gln Gln Gly Pro Gly Gln		
245	250	255
Gln Gly Pro Gly Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala		
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Ser Ala Ala Ser Gly Gly Tyr Gly Pro Gly Ser Gly Gln Gln Gly Pro		
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Gly Gln Gln Gly Pro Gly Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser		
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Ala Ala Ala Ala Ala Ala Gly Gly Tyr Gly Pro Gly Ser Gly Gln Gln		
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Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly		
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Pro Gly Gly Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala		
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Ala Ala Gly Gly Tyr Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Gln		
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Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln		
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Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly		
385	390	395
Pro Gly Gln Gln Gly Pro Gly Gly Gln Gly Ala Tyr Gly Pro Gly Ala		
405	410	415

Ser Ala Ala Ala Gly Ala Ala Gly Gly Tyr Gly Pro Gly Ser Gly Gln
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Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln
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Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly Pro Gly Gln Gln Gly
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Pro Gly Gln Gln Gly Pro Tyr Gly Pro Gly Ala Ser Ala Ala Ala Ala
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Ala Ala Gly Gly Tyr Gly Pro Gly Ser Gly Gln Gln Gly Pro Gly Gln
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Gln Gly Pro Gly Gln Gln Gly Pro Gly Gly Gln Gly Pro Tyr Gly Pro
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Gly Ala Ala Ser Ala Ala Val Ser Val Gly Gly Tyr Gly Pro Gln Ser
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Ser Ser Val Pro Val Ala Ser Ala Val Ala Ser Arg Leu Ser Ser Pro
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Ala Ala Ser Ser Arg Val Ser Ser Ala Val Ser Ser Leu Val Ser Ser
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Gly Pro Thr Lys His Ala Ala Leu Ser Asn Thr Ile Ser Ser Val Val
 565 570 575

Ser Gln Val Ser Ala Ser Asn Pro Gly Leu Ser Gly Cys Asp Val Leu
 580 585 590

Val Gln Ala Leu Leu Glu Val Val Ser Ala Leu Val Ser Ile Leu Gly
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Ser Ser Ser Ile Gly Gln Ile Asn Tyr Gly Ala Ser Ala Gln Tyr Thr
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Gln Met Val Gly Gln Ser Val Ala Gln Ala Leu Ala
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<210> 9

<211> 410

<212> PRT

<213> Araneus diadematus

<400> 9

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Gly Gly Pro Val Ser Ser Ala Ala Ala Ala Ala Ala Ala Gly Ser Gly
 35 40 45

Pro Gly Gly Tyr Gly Pro Glu Asn Gln Gly Pro Ser Gly Pro Gly Gly
 50 55 60

Tyr Gly Pro Gly Gly Ser Gly Ser Ser Ala Ala Ala Ala Ala Ala Ala
 65 70 75 80

Ala Ser Gly Pro Gly Gly Tyr Gly Pro Gly Ser Gln Gly Pro Ser Gly
 85 90 95

Pro Gly Gly Ser Gly Gly Tyr Gly Pro Gly Ser Gln Gly Ala Ser Gly
 100 105 110

Pro Gly Gly Pro Gly Ala Ser Ala Ala Ala Ala Ala Ala Ala Ala Ala
 115 120 125

Ala Ser Gly Pro Gly Gly Tyr Gly Pro Gly Ser Gln Gly Pro Ser Gly
 130 135 140

Pro Gly Ala Tyr Gly Pro Gly Gly Pro Gly Ser Ser Ala Ala Ala Ala
 145 150 155 160

Ala Ala Ala Ala Ser Gly Pro Gly Gly Tyr Gly Pro Gly Ser Gln Gly
 165 170 175

Pro Ser Gly Pro Gly Val Tyr Gly Pro Gly Gly Pro Gly Ser Ser Ala
 180 185 190

Ala Ala Ala Ala Ala Ala Gly Ser Gly Pro Gly Gly Tyr Gly Pro Glu
 195 200 205

Asn Gln Gly Pro Ser Gly Pro Gly Gly Tyr Gly Pro Gly Gly Ser Gly
 210 215 220

Ser Ser Ala Ala Ala Ala Ala Ala Ala Ala Ser Gly Pro Gly Gly Tyr

225					230					235					240		
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385					390					395					400		
Ser	Thr	Gln	Met	Ile	Ser	Gln	Ala	Leu	Ser								
				405					410								

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Ser Ala Pro Val Ala Ser Ala Ala Ala Ser Arg Leu Ser Ser Pro Ala
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Ala Ser Ser Arg Val Ser Ser Ala Val Ser Ser Leu Val Ser Ser Gly
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Pro Thr Asn Gln Ala Ala Leu Ser Asn Thr Ile Ser Ser Val Val Ser
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Gln Val Ser Ala Ser Asn Pro Gly Leu Ser Gly Cys Asp Val Leu Val
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Gln Ala Leu Leu Glu Val Val Ser Ala Leu Val Ser Ile Leu Gly Ser
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<210> 11

<211> 125

<212> PRT

<213> Artificial sequence

<220>

<223> NR4 (ADF-4)

<400> 11

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Leu Ser Ser Pro Ala Ala Ser Ser Arg Val Ser Ser Ala Val Ser Ser
 35 40 45

Leu Val Ser Ser Gly Pro Thr Asn Gly Ala Ala Val Ser Gly Ala Leu
 50 55 60

Asn Ser Leu Val Ser Gln Ile Ser Ala Ser Asn Pro Gly Leu Ser Gly
 65 70 75 80

Cys Asp Ala Leu Val Gln Ala Leu Leu Glu Leu Val Ser Ala Leu Val
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Ala Ile Leu Ser Ser Ala Ser Ile Gly Gln Val Asn Val Ser Ser Val
 100 105 110

Ser Gln Ser Thr Gln Met Ile Ser Gln Ala Leu Ser Gly
 115 120 125

<210> 12

<211> 1959

<212> DNA

<213> Araneus diadematus

<400> 12

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<210> 13

<211> 2013

<212> DNA

<213> *Araneus diadematus*

<400> 13

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<210> 14

<211> 420

<212> DNA

<213> Artificial sequence

<220>

<223> NR3 (ADF-3)

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<210> 15

<211> 375

<212> DNA

<213> Artificial sequence

<220>

<223> NR4 (ADF-4)

<400> 15

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<210> 16

<211> 2828

<212> DNA

<213> Nephila clavipes

<400> 16

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<211> 2724

<212> DNA

<213> *Nephila clavipes*

<400> 17

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35 40 45

Gln Ile Asn Glu Val Asp Thr Ser Gly Pro Gly Gln Tyr Tyr Arg Ser
50 55 60

Ser Ser Ser Gly Gly Gly Gly Gly Gln Gly Gly Pro Val Val Thr
65 70 75 80

Glu Thr Leu Thr Val Thr Val Gly Gly Ser Gly Gly Gly Gln Pro Ser
85 90 95

Gly Ala Gly Pro Ser Gly Thr Gly Gly Tyr Ala Pro Thr Gly Tyr Ala
100 105 110

Pro Ser Gly Ser Gly Ala Gly Gly Val Arg Pro Ser Ala Ser Gly Pro
115 120 125

Ser Gly Ser Gly Pro Ser Gly Gly Ser Arg Pro Ser Ser Ser Gly Pro
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Ser Gly Thr Arg Pro Ser Pro Asn Gly Ala Ser Gly Ser Ser Pro Gly
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Gly Ile Ala Pro Gly Gly Ser Asn Ser Gly Gly Ala Gly Val Ser Gly
165 170 175

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Met Leu Ser Gln Tyr Ser Ser Gly Ser Gly Thr Cys Asn Pro Asn Asn
 35 40 45

Val Asn Val Leu Met Asp Ala Leu Leu Ala Ala Leu His Cys Leu Ser
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Asn His Gly Ser Ser Ser Phe Ala Pro Ser Pro Thr Pro Ala Ala Met
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ggcggtgcg gcggtccgta c 81

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1 5 10 15

Asp Gly Pro Ile Thr Ile Ser Glu Glu Leu Thr Ile
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<210> 44

<211> 36

<212> DNA

<213> Artificial Sequence

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<223> PCR-Primer

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gaaaagaagc tttcattagc ctgggctgta tgggtcc

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<210> 45

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<212> DNA

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<211> 42

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Recombinant spider silk proteins

1. A recombinant spider silk protein comprising
 - a) one or more synthetic repetitive spider silk protein sequences, and / or
 - b) one or more authentic non-repetitive spider silk protein sequences.
2. The recombinant spider silk protein of claim 1, wherein the sequences are derived from spider dragline proteins from the major ampullate gland and/or from proteins derived from the flagelliform gland.
3. The recombinant spider silk protein of claim 1 or 2, wherein the authentic non-repetitive sequences are derived from the amino terminal non-repetitive region (flagelliform proteins) and/or the carboxy terminal non-repetitive region (flagelliform and dragline proteins) of a naturally occurring spider silk protein.
4. The recombinant spider silk protein of claim 3, wherein the authentic non-repetitive sequence is modified in order to adapt said sequence to expression in a host and preferably is SEQ ID NO: 14, 15, 32 and 34 or a variant thereof.
5. The recombinant spider silk protein of one or more of claims 1 – 4, wherein the dragline and/or flagelliform proteins are selected from dragline or flagelliform proteins of orb-web spiders (Araneidae).
6. The recombinant spider silk protein of claim 5, wherein the dragline proteins are derived from *Araneus diadematus* and the flagelliform proteins are derived from *Nephila clavipes*.

7. The recombinant spider silk protein of one or more of claims 1-6, wherein the dragline proteins are wild type ADF-3, ADF-4, MaSp I or MaSp II and the flagelliform protein is FLAG.
8. The recombinant spider silk protein of one or more of claims 1-6, wherein the dragline protein is ADF-3 encoded by SEQ ID NO:1 and/or ADF-4 encoded by SEQ ID NO:2 or the flagelliform protein is SEQ ID NO: 6 (Flag-N) and/or SEQ ID NO: 7 (Flag-C), or a variant thereof.
9. The recombinant spider silk protein of one or more of claims 1-8, wherein the synthetic repetitive sequences comprise one or more polyalanine containing consensus sequences.
10. The recombinant spider silk protein of claim 9, wherein the polyalanine containing consensus sequence is derived from ADF-3 and has the amino acid sequence of SEQ ID NO: 3 (module A) or a variant thereof.
11. The recombinant spider silk protein of one or more of the preceding claims, wherein the synthetic repetitive sequence is derived from ADF-3 and comprises one or more repeats of the amino acid sequence of SEQ ID NO: 4 (module Q) or a variant thereof.
12. The recombinant spider silk protein of one or more of claims 9-11, wherein the synthetic repetitive sequence comprises one or more of (AQ) and/or (QAQ) as repeat units.
13. The recombinant spider silk protein of claim 12, wherein the synthetic repetitive sequence is (AQ)₁₂, (AQ)₂₄, (QAQ)₈ or (QAQ)₁₆.
14. The recombinant spider silk protein of one or more of claims 1-13, wherein the synthetic repetitive sequence is derived from ADF-4 and comprises one or more repeats of the amino acid sequence of SEQ ID NO: 5 (module C) or a variant thereof.

15. The recombinant spider silk protein of claim 14, wherein the synthetic repetitive sequence is C₁₆ or C₃₂.
16. The recombinant spider silk protein of one or more of the preceding claims, wherein the synthetic repetitive sequence is derived from a flagelliform protein and is module K (SEQ ID NO: 35), module sp (SEQ ID NO: 37), module X (SEQ ID NO: 39), and module Y (SEQ ID NO: 41).
17. The recombinant spider silk protein of claim 16, which is Y₈, Y₁₆, X₈, X₁₆, K₈, or K₁₆.
18. The recombinant spider silk protein of one or more of the preceding claims wherein the complete recombinant spider silk proteins comprise formula (QAQ)₈NR₃, (QAQ)₁₆NR₃, (AQ)₁₂NR₃, (AQ)₂₄NR₃, C₁₆NR₄ or C₃₂NR₄, (QAQ)₈, (QAQ)₁₆, (AQ)₁₂, (AQ)₂₄, C₁₆ or C₃₂.
19. A nucleic acid sequence, coding for a recombinant spider silk protein of one or more of claims 1-18.
20. A vector, which comprises the nucleic acid sequence of claim 19 and which preferably is derived from the cloning vector of Fig. 6 or SEQ ID NO: 55 or a variant thereof.
21. An expression vector, which comprises the nucleic acid sequence of claim 19 and one or more regulatory sequences.
22. The vector of claims 20 or 21, which is a plasmid or a viral vector, preferably a baculovirus system or a vaccinia virus vector system.
23. A host, which has been transformed with the vector of any of claims 20-22.
24. The host of claim 23, which is a prokaryotic cell.
25. The host of claim 24, which is *E.coli* or *Bacillus subtilis*.

26. The host of claim 25, which is a eukaryotic cell.
27. The host of claim 26, which is a mammalian cell, plant cell, yeast cell or an insect cell.
28. The mammalian cell of claim 27, which is a CHO, COS, HeLa, 293T, HEH or BHK cell.
29. The host of claim 27, which is a yeast cell.
30. The host of claim 29, which is *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Candida albicans*, *Hansenula polymorpha*.
31. The host of claim 27, wherein the insect cell is selected from Lepidoptera insect cells, preferably from *Spodoptera frugiperda* and from *Trichoplusia ni*.
32. The host of claim 31, wherein the insect cell is a Sf9, Sf21 or high five cell.
33. The host of claim 27, wherein the plant cell is derived from tobacco, potato, corn, pea and tomato.
34. A method of aggregation of spider silk proteins, comprising the following steps:
 - a) preparing a protein solution containing unoriented spider silk proteins as defined in one or more of claims 1-17;
 - b) exposing the solution prepared in a) to an aggregation trigger; and
 - c) recovering the precipitated spider silk proteins.
35. The method of claim 34, wherein the spider silk proteins used in step a) are produced by transforming a suitable host of one or more of claims 23 to 33 with a vector of claim 20-22 or a nucleic acid of claim 19, and expressing the spider silk protein under suitable conditions.

36. The method of claim 34 or 35, wherein the aggregation trigger is selected from acidification, preferably to a pH of about 1, potassium phosphate and mechanical stress, preferably rotating the protein solution and applying shearing forces.
37. The method of one or more of claims 34-36, which further comprises the step of spinning said proteins provided in step a) or recovered in step c) into filaments, nanofibers and threads by a suitable method or forming a film.
38. Use of the proteins/threads as defined in one or more of the preceding claims in the field of biotechnology and/or medicine.
39. Use of the proteins/threads as defined in one or more of the preceding claims for the manufacture of wound closure or coverage systems.
40. The use of claim 39 for the manufacture of suture materials.
41. The use of claim 40, wherein the suture material is intended for use in neurosurgery or ophthalmic surgery.
42. Use of the proteins/threads as defined in one or more of the preceding claims for the manufacture of replacement materials, preferably artificial cartilage or tendon materials.
43. Use of the proteins/threads as defined in one or more of claims 1-17 in the manufacture of automotive and aircraft parts.
44. Wound closure or coverage systems, suture materials, replacement materials, preferably artificial cartilage, tendon materials, automotive parts or parts used in the aircraft construction, which comprise a thread/protein of one or more of claims 1-17 or which are obtainable by a method of one or more of claims 33-36.

45. A paper product, comprising a recombinant spider silk protein of one or more of claims 1-17.
46. A textile or leather product, comprising a recombinant spider silk protein of one or more of claims 1-17.
47. The textile or leather product of claim 45, wherein the recombinant spider silk protein is present as a coating.
48. A gel or a foam comprising or consisting of a protein of one or more of claims 1-17.
49. The gel of claim 48, which comprises or consists of proteins based on (QAQ)₈NR₃, (QAQ)₁₆NR₃, (AQ)₁₂NR₃, (AQ)₂₄NR₃, C₁₆NR₄ or C₃₂NR₄, (QAQ)₈, (QAQ)₁₆, (AQ)₁₂, (AQ)₂₄, C₁₆ or C₃₂.
50. Coatings for implants and stents comprising or consisting of a protein of one or more of claims 1-17.
51. A thread or fiber, comprising a protein/thread of one or more of claims 1-17 and a further fiber, the fiber not being of spider origin and preferably being a plant derived fiber or a synthetic fiber.
52. A film comprising or consisting of a protein of one or more of claims 1-17 or which is based on (QAQ)₈NR₃, (QAQ)₁₆NR₃, (AQ)₁₂NR₃, (AQ)₂₄NR₃, C₁₆NR₄ or C₃₂NR₄, (QAQ)₈, (QAQ)₁₆, (AQ)₁₂, (AQ)₂₄, C₁₆ or C₃₂.
53. The film of claim 52, which comprises or consists of proteins based on (AQ)₂₄NR₃ or C₁₆.

54. The film of claim 52 or 53, wherein the film surface is modified by small organic molecules and/or biological macromolecules, for example proteins, fluorescein or β -galactosidase.