The complexity of silk under the spotlight of Synthetic Biology

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Abstract

For centuries silkworm filaments have been the focus of R&D innovation centred on textile manufacture with high added value. More recently, silk research has focussed on more fundamental issues concerning bio-polymer structure-property-function relationships. This essay outlines the complexity and fundamentals of silk spinning, and presents arguments for establishing this substance as an interesting and important subject at the interface of Systems Biology (discovery) and Synthetic Biology (translation). It is argued that silk is a generic class of materials where each type of silk presents a different embodiment of emergent properties that combine genetically determined (anticipatory) and environmentally responsive components. In spiders' webs the various silks have evolved to form the interactive components of an intricate fabric that provides an extended phenotype to the spider's body morphology.

Introduction

Silk has for some time been one of the much heralded success stories of Bio-Engineering with dope expression in potatoes [1] various microbes [2] and even goat-milk [3] some with names like Bio-Steel ™ to market the fibrous product. Yet none of these products is real silk, as this essay shall propose; nor do any of the filaments so far seem to match the ecological footprint of spider dragline fibres even if material characteristics are apparently beginning to approach the model material [4]. But the quest is on, and new commercial brands are being created around the concept of silk-derived, eco-friendly filaments with great mechanical properties and comparable durability. An interesting and highly relevant example is the efforts of Bolt Threads in California, which is developing novel approaches to the problem of extraction-denaturation of the protein by using yeasts to express and expel its silk-inspired dope molecules. The example is doubly relevant to the subject of this essay as a recent article headed 'Tech investors bet on synthetic biology' in London's *Nature* listed 7 specific, high-value and high-profile SynBio investments [5]. One of these investments, underwritten by 2 co-founders of PayPal, was close on $40M into Bolt Threads and its 'silks'.

Good bio-polymer threads are being produced, I am sure, and will probably be produced in the long run also economically, using advanced technologies and genetically engineered poly-peptides whether the carrier organism is a yeast, microbe, potato or mammal [6]. However, are the threads spun from these dopes really silk? Indeed: What's in a name? In 1951 a German court (Oberlandesgericht Freiburg) ruled against Bayer's use of the term 'Seide' (Silk) in its branding of 'Kunstseide' (Artificial Silk) because, so the ruling went: *silk* is a very specific natural product with particular properties, and Bayer was seen to be trying to cash in on the name of a well respected generic 'brand' name [7]. Is this issue still germane today, too, for some of the new artificial silks? After all, the reason for the many enterprises using the words of *spider* and *silk* (often in combination) are hoping to cash-in on the implicit association with something superior, luxurious and sustainable.

However that might be, natural silk is indeed special and this essay will outline both the reasons for this statement and its relevance for the budding field of Synthetic Biology. Silk, for starters, is not a relatively simple cellular product like, for example, an enzyme where the rule of 'one gene-one protein' might just apply. Silk, in contrast, is the product of a complex multi-component system with layers of molecular interactions before, during and after their integration into one material. This creates inherent complexity and emergent properties resulting from the interaction of many genes active in different tissues compounded by the effects of strong external components with factors not controlled tightly (if at all) by the organism. All silks function outside the animal's body, often for months, and have evolved in response to selection on a layer of levels that led to the many details of structural and functional complexity of the spider's web. And in this context of a life-dinner arms race between insect prey and spider predator, silks are a key component in the Extended Phenotype [8] of the gossamer trap that expands the spider's reach. The spider's web-building behaviour shapes a snare that is only possible because of the silken threads, glues and cements; and the selection on the combined structure by the prey trying to escape is as much on the ensnaring material as it is on the construction behaviour.

What is a Silk?

Let me briefly outline the biological singularity of the material 'Silk'. Natural silk is a class of protein-based poly-mer materials that have evolved several times independently in the arthropods [9]. By definition, a silk is extrusion *spun* through a duct and nozzle from a dope that has been stored for various lengths of time in a gland. In contrast to simply discharging from a gland, spinning allows the animal to manage the extrusion process and its logistics by tightly controlling the addition of chemicals and energy and thus steer the conversion of the core components from liquid dope to solid fibre [10]. It is this extrusion process that sets silks apart from all other complex biological materials, which are *grown* inside or outside a cell in the process of modular assembling. Growing is relatively slow when compared to spinning, where the molecular modules are not stacked but simply rearranged to allow the microsecond conversion from liquid to solid [10]. Clearly, any analytical and manufacturing approach to silks

has to take inspiration not only from the silk molecules but also from these sol-gel conversions and phase transitions. Importantly, industry has been extrusion-spinning polymers for a very long time and in the process has developed dedicated and highly sophisticated tools of polymer physics [11,12].

This combination of well understood industrial spinning (of synthetic polymers) and little understood natural spinning (of silk bio-polymers) could bring a new dimension to silk research (in the context of Synthetic Biology) by looking beyond the intra-cellular processes towards the even more complex processes of predictive extra-cellular assembly. These processes can be called predictive or anticipatory because the components are outside any direct cellular control with the molecular anticipation providing the very basis for properties emergence. Indeed, the whole assembling process has to rely on all the individual components acting in unison (and with the molecules behaving largely to form) in order to efficiently and effectively build the supra-molecular structure of the silk thread. Of course, as in the case of an extended phenotype, it is this final outcome of the *whole process* that is judged by selection, which in turn over evolutionary millennia has fed-back piecemeal yet interactively into each and all of the individual steps of the production process.

It is in this understanding of the overall integration of components that we must call any silk the phenotypic extension of a complex network of genetic determinants acting and controlling each step of a process that relies on emergent properties. In a spider the process of silk production would go something like this: it starts with (i) the assembly of the molecules inside the cell followed by their (ii) packaging and transport to the cell surface where they are (iii) stored in the lumen of the gland to then be (iv) transported towards the gland exit and duct interface where the molecules are then (v) unpacked to be able to (vi) elongate and interact in the down-flow through a tapered funnel into a narrow duct where they then (vii) assemble into a thread that then, in its draw-through the duct, is (viii) further dehydrated and post-drawn while being (ix) coated with layers of protective or bio-functional compounds before the (x) water coat is stripped by the spinneret nozzle and (xi) the filament, now in the ambient air, is further draw-stretched while drying out further [10].

Silk and SynBio?

The advocacy of seeing silk not with the Bio-engineering spectacle of 'one-gene one-protein' but viewing silk as an extended phenotype building on 'many genes – several proteins – and a complex process to follow' should be of great interest to Synthetic Biology. Why? Perhaps this is the time to define/describe Synthetic Biology in the context of silk R&D? Slightly paraphrased, the NEST Expert Group calls Synthetic Biology (from now-on called SynBio) : "… the synthesis of complex, biologically, based (or inspired) systems displaying functions not existing in Nature. This engineering perspective may be applied at all levels of the hierarchy of biological structures, from individual molecules to whole cells, tissues and organisms. In essence, synthetic biology will enable the design of, 'biological systems' in a rational and systematic way.' [13].

This view of its subject and mission makes SynBio not only a powerful tool for genetic manipulations on a large scale (much grander than performed under the old Genetic- or Bio-Engineering concept) but also an effective instrument to test hypotheses about a wide range (simple to complex) of biological processes and materials. In this, SynBio has a much broader remit than Bioengineering. In the context of silk this distinction between the two fields would mean that 'silk protein-motifs spliced into another organism's genome' would not, in itself, be Synthetic Biology whether such genomes come from microbes, animals or plants. And in this spirit of fundamental protein research leading to novel bio-polymer materials development: silk has much more to offer to SynBio (and human-kind) than a library silk motifs that may - or may not - be of dubious or significant relevance to fibre properties [14].

Implicit in the science (and definition) of SynBio is a definite progress from fundamental inventions (based on research) leading to marketable innovations (based on more research). In this goal SynBio relies to some extent on its sister field of Systems Biology, which by most definitions focuses on providing fundamental data gleaned from complex biological systems. Thus Systems Biology greatly enhances the power of SynBio if we see SynBio not only as a tool for pragmatic R&D but also as an experimental selection arena for the testing of specific hypotheses on complex biological interactions i.e. invention leading to innovation.

Along this line of thinking, silks can provide the basis, and make a powerful argument, for a concerted SynBio effort towards the goal of an overall bio-inspired manufacture of a novel class of complex materials all the way from molecular assembly to supra-molecular arrangement. Of course, as indicated, such effort would have to be accompanied by comparable efforts using the tools of Systems Biology to help us understand the fundamental biology of the material and its genesis.

Real silks, unlike their semi-synthetic copies, are much more than a repetitive sequence of simple peptide motives bracketed by end groups. Let me list key features that will later be revisited in more detail. (A) Most silks have two large principal proteins consisting of many repetitions of specific short motifs interspersed by different motifs. (B) Silks have evolved independently a number of times, with some spider and silkworm filaments showing evolutionary convergence. (C) Silks cover a wide diversity in the details of the motifs that in a modular fashion make up the repetitive or disruptive mers of the silk bio-polymer. (D) Silks, by definition, are spun, with some motifs probably relating to safe pre-spinning storage, others to spinning conversions and yet others to filament properties. (E) The transition from one form of silk to another (dope to fibre) is energy efficient and we may assume that this predictive transformation is also somehow expressed in the sequence. (F) At present we understand very little about any of the underlying structure-property-function relationships of even the best studied of silks.

This brief simplification of the intricacy of silk structure-property-function relationships will have given a first insight into the complexity of the system 'silk

& spinning' and hopefully also illustrated the reasons for viewing the web/silk combination as an extended phenotype. The overview will now be expanded with more details that hopefully will lead to potential research questions. In the context of the advocacy, which is the basis of this essay, questions tend to be better motivators than statements, not least when the statements would be largely hypothetical and without much support by a solid body of fundamental research. After all, presently we have still many open questions about the details of most key components, drivers and processes in the natural silk production process, despite much research and progress and despite generally positive views of our progress in copying aspects of the sequences in order to make outstanding filaments and other products [2,15,16]. Indeed, this overall lack of full understanding provides a powerful motive and strong incentive for R&D on Silk using Synthetic Biology which, by the definition given above, aims to create complex functional structure-property relationships semi-synthetically in biological systems.

Silks as a powerful paradigm and test for the SynBio approach?

Here a preamble is necessary. The field of silk-research is expanding very rapidly, with many diverging, and often contradictory, conclusions arising from diverse experiments, technologies, measurements and even definitions. This makes it impossible, without over-extensive and probably also highly biased pre-selection, to provide comprehensive and fair referencing. Hence I leave it to the reader interested in following-up on specific features of silks in the context of SynBio hypothesis-testing to consult the wider 'web' for relevant publications and take his or her more focussed literature research from there.

(A) Why would it be that there are typically two principal proteins involved in what is considered a 'typical' silk such as the dragline of a spider or the cocoon thread of a silkworm? Of course there are many exceptions such as for example the alpha silks of the hymenoptera [18] or the multi-protein silks of the chironomids [19]. But the benchmark silks of the domesticated and wild silkworms or the araneid spiders all have one larger and one shorter protein, with both seemingly necessary to interact and combine somehow to make for a good silk, with the addition, of course, of considerable hierarchical structure [20]. Some arguments suggest that the interaction of a long, largely hard, and a shorter, largely soft, protein form a good combination to make a molecular composite that is both strong and extensible i.e. tough [16,21,22]. Moreover, the interaction of two such molecules could allow one molecule to block-off certain folding patterns in the other. Either way, the nano-scale structure of silk on the molecular and supra-molecular level could be compared to the micron-scale structure of biological materials such as nacre and bone where the combination of two mediocre compounds makes for an outstanding composite [23]. The key difference is the fact that in silk the components are spun together, not grown layered or into each other as in these other composite materials [24]. An important hypothesis testable by SynBio would address the interactions and contributions to the material properties of the large/heavy chain and the smaller/light chains in both spider dragline and mulberry/wild-worm cocoon silks.

(B) The independent evolution of silk filaments in the insects, the arachnids and the crustaceans is a strong indication of a good (i.e. cost-benefit positive) solution that is highly energy efficient. In spiders there seems to be a strong link between the structural complexity of the spinning device and the ratio of a key amino acid, Gly, which in turn can be an indicator of folding complexity of the molecule that contains much of Gly in dedicated motifs [25]. (FIGURE 1).

However, silks have not only evolved independently in the insects and spiders but also have even converged in molecular sequence to an astounding degree in various unrelated species belonging to different taxa [27]. Silks have also evolved in glorious examples of adaptive radiation within each of the major taxa so that there are a number of insect orders other than moths (Lepidoptera) that make silks, and also a number of silk spinning arachnid orders other than spiders (Araneae). Most interestingly for detailed SynBio hypothesis-testing is the observation that within the higher web-building spiders, the original ancestral silk gland has radiated into a set of very different glands that spin a diverse range of silks from a varied range of dope chemistries using a wide range of duct anatomies. As these glands are without doubt homologous, they provide a superb platform for SynBio to tackle specific hypotheses on structure-property-function relationships in silks.

(C) There are many different silks found in Nature (there should be well over 100'000 types if we consider also small inter-specific differences), which has created a wealth of information that can be probed for 'meaning' by analysing its encoded structure-function instructions. Overall the number of motifs, which in a modular fashion make up the mers, seems limited. However, in combination with the motif's position in the polymer sequence even a small number of motifs provide a huge number of options for adaptive radiation. Especially if the polymer sequence is long, which it is in the case of most silks. Simply shuffling the motif-mers around in the sequence will have implications on the folding of the polymer protein; adding new motifs and increasing their frequency can add exponentially to the complexity.

Moreover, spin-technology is adding further options available to adaptive radiation. As already mentioned, silks are not only liquid dopes, they must be spun into filaments to fulfil their full function. Thus the spinning process also contributes significantly towards the ultimate material properties, which are then evaluated by Nature's selection process. The great importance of spinning conditions on thread properties has been shown for both spider and silkworm silks, where (for example) simple changes in the spinning speed and the animal's body temperature can have huge effects on the stress strain curves (and thus the toughness profile) of the filaments [28]. Post-spinning draw-down conditions add to the complexity of the process by adding significantly to the overall material properties [29]. Of course, natural selection works on the filaments, which in turn feeds-back to both molecular composition and spinning conditions. Alas, to date we are still very much in the dark about the details of the relevant drivers. It would be another interesting challenge for the SynBio approach to test how one could design silk-like polymers by fine-tuning each component: (a) the spinning

dope, (b) the extrusion process and (c) the draw-down. Such informed design could have enormous implications for the manufacture of future sustainable polymers.

(D) It will have become amply apparent by now that silks are intriguing bio-polymers that combine energy efficient dope manufacture with its safe storage to be followed by energy efficient extrusion into a filament that at emergence from the spigot is still pliable enough to respond well to post-spin draw-down. Once spun, a filament can last (and function) for days, weeks, months or even years in the open environment with all its abiotic challenges to the inert protein. Even bacteria and fungi seem to find it very hard to digest a silk judging from the many webs that can last months without apparent loss of function. Thus, in silks, evolution has provided us with a class of model materials covering many of the challenges faced by synthetic polymers.

One major difference between silks and manmade polymers is the diversity of motifs and of their arrangements in the silks and the absence of molecular diversity in synthetic polymers. This diversity and its role is something that SynBio could address very competently by testing hypotheses on the various motifs and their effects on the properties of the bulk material. Here we may also remember the largely unresolved roles of each of the two principal component polymer molecules so typical for silks but absent in synthetic polymers. Perhaps SynBio will be able to elucidate routes to making better and truly bio-inspired polymers?

Silk spinning is principally the folding of a set of protein molecules into a composite with different levels of complexity. The behaviour of the molecules is encoded in the molecular sequence but other factors, such as post-spinning draw-down, play significant roles in the overall process and the performance of its end-products [29]. Of potential relevance for understanding silk protein folding could be particular parallels to amyloids and their formation [26]. However, in the case of the silk the molecular states have evolved under natural selection for controlled folding while in the case of the amyloid they are, as far as we know, an unintended consequence of uncontrolled folding. An interesting set of hypothesis ready for SynBio testing in the search to un-fold Amyloids in vivo would build on insights gained from silk formation and the crucial instability inter-face during the sol-gel transition.

(E) Many silks not only make outstanding filaments – but also seem to do so surprisingly efficiently. While the real costs of the acquisition of the protein precursors and the amino acid assembly into the silk molecules has not been calculated as yet, the costs of con-gelling the liquid molecules into a solid polymer have been studied experimentally [30,31]. Surprisingly, the animal uses melt-spinning technology with the silk-dope melt-flowing at ambient temperatures. The trick used here is the tight integration of the water-molecule into the protein molecule. This allows the material to behave in a Non-Newtonian and liquid-crystalline manner despite high protein concentrations, and convert very simply from the aqueous phase to the filamentous phase simply by expelling the water molecules. This folding phase-transition occurs early in

the duct and is facilitated by a small but significant pH shift which leads to a large-scale reformatting of H-bonds from Amide-Water-Amide to Amide-Amide bonding [32].

This process, which was named Aquamelt spinning because of the key role of water mobility, allows a silk to be spun at energy inputs a thousand times lower than the energy required to spin a synthetic HDPE polymer [31]. SynBio tools would be extremely useful in further illuminating and testing specific processes involved in the aquamelt protein refolding. Once again evolution has provided us with processes and materials that can be practical models for many of the challenges faced by synthetic polymers, especially when exploring environmentally friendly manufacture options.

(F) As will have become apparent by now, at present we have huge gaps in our understanding of the underlying structure property function relationships in silks, despite more than a hundred years of research progressing from key work by Astbury and Linus Pauling in the 30ties and 40ties and rapidly growing at the beginning of this century [33,16]. Important for this research was always the simple fact that silk proteins provide a most fertile field in a readily accessible material that can be tested rather easily both in vitro and in vivo [34, 35].

Of importance for the next steps in silk research will be more sophisticated hypothesis testing and the translation of discoveries and resulting inventions into marketable innovations. And here the field of SynBio could really make a difference by developing insights gained from silks into testable models for the design of novel polymers. For that to happen a diverse range of biological processes related to natural silk production has to be understood, tested and reduced to practice.

Questions have to be asked from both Systems Biology and Synthetic Biology - to be followed up in some cases by Genetic-/Bio-Engineering. There can be questions addressing silk molecular composition: What, in principle, determines a good spinning dope? More specifically: What is the role of the various motifs (for example the RGDs in Antherea silks) in the structure-function equation of a particular silk? There can also be questions addressing the sol-gel phase transition of the protein melt: How does the minute shifts in pH control the denaturation transition and formation of particular silk proteins? What is the importance of the terminal groups and their adjacent motifs for the transitions from highly re-active precursor (Silk I) in the dope to the largely in-active (Silk II) conformation of the thread? How does the flow-elongation in the duct affect molecular orientation, alignment and interlinking? What is the relative importance of inter-and intra-molecular bonding for the material's integrity and behaviour? Indeed, what is the importance of molecular weight and conformation for silk properties?

Conclusions

Hopefully this essay will have given sufficient fundamental background to generate deeper interest in the many aspects of silk's potential and relevance for

the SynBio community. Hopefully the examples and questions will also have supported the point made in the introduction: namely that expression of silk-like peptides in another organism followed by extrusion through a simple hole-plate is not sufficient for making a proper bio-mimetic silk-filament, however acceptable the material properties of such fibres might be. The challenges to Synthetic Biology will be in the uncovering as well as the testing of key principles underlying the molecular structures of as many silks as possible, their folding and phase transitions and their final structural properties.

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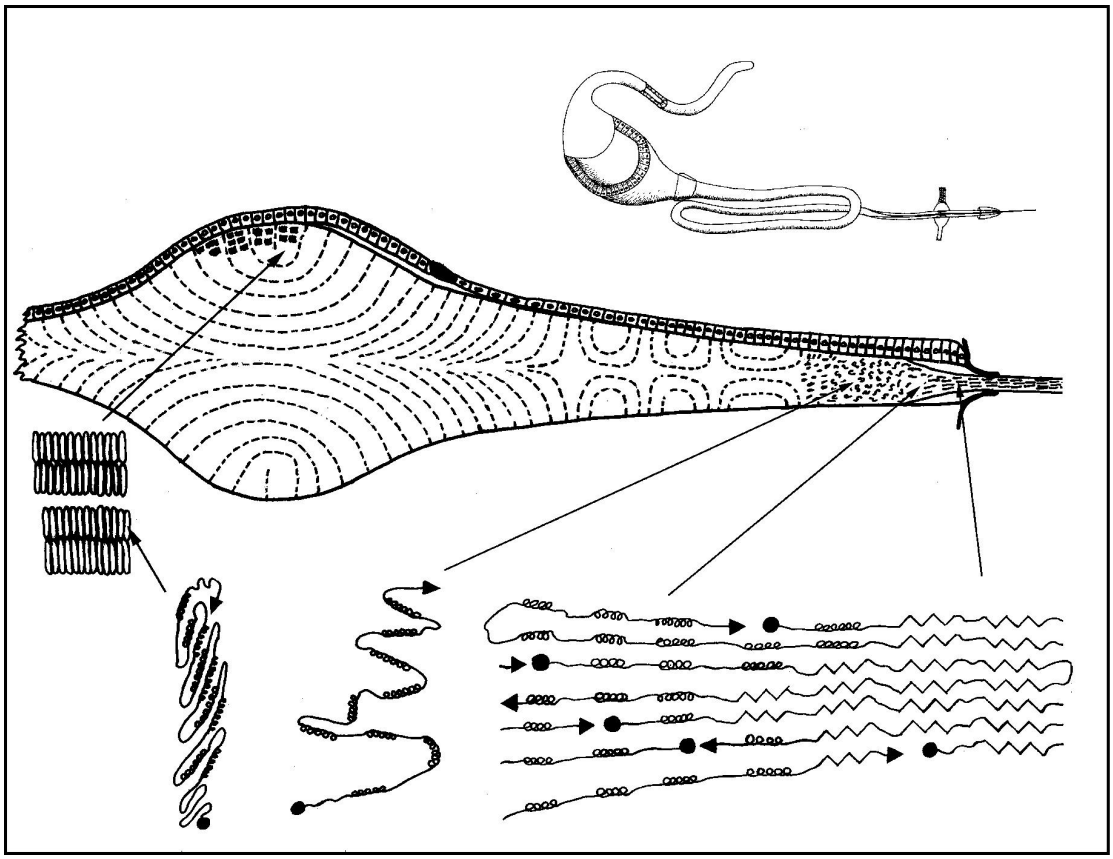

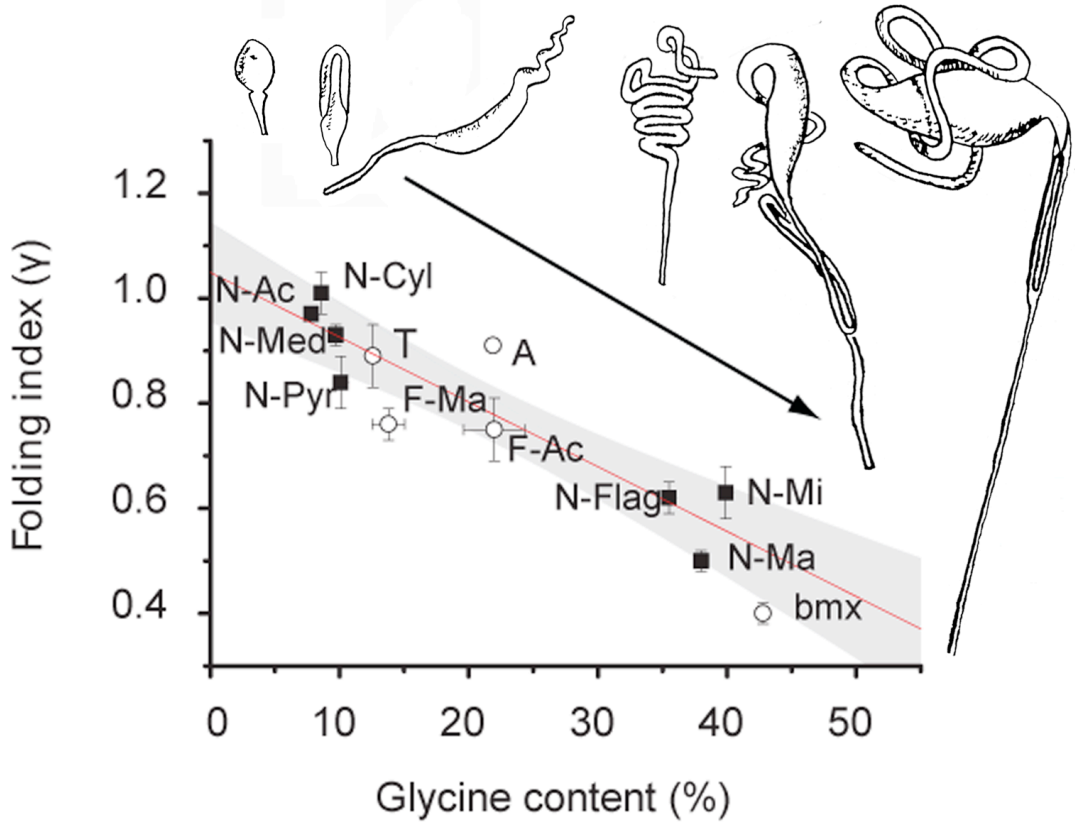

Figure 1 The interaction of spider silk dope chemistry and complexity of spinning process. Shown is the relationship between the Glycine content of a silk and the 'folding index' γ of the silk-proteins in the pre-spun liquid stage with γ being taken to be indicative of the protein intrinsic disorder. The correlation and model are taken to quantitatively explain the structure-function relationship by describing the molecular conformation i.e. the β-sheet propensity. The data suggest that, in order to achieve specialization and performance, silks require higher structural flexibility at the expense of reduced stability and increased conversion energy. A γ value near 1 would denote helix-type folding while γ values <0.5 would signify mostly unfolded chains having been calculated from the ratio of the circular dichroism spectrum bands at 208nm and 220nm (at 20°C). The arrow show the direction of gland evolution and the insets depict schematically the overall gland shape (not to scale). The regression was calculated from dopes of the 7 different gland types of a *Nephila* spider and analyzed by a general linear model (GLM). The correlation was tested with spider dopes from other spiders, only very distantly related and typically much more ancestral. The out-group was the mulberry silk worm *Bombyx mory* (bmx), which has evolved silk independently of the spiders. Legend: *Nephila edulis* (Tetragnathidae) and its different glands Major ampullate (N-Ma), Minor ampullate (N-Mi), Flagelliform (NFlag), Cylindriform (N-Cyl), Aciniform (N-Ac), Pyriform (N-Pyr) and Median (N-Med). Another web spider *Kukulkania hibernalis* (Filistatidae) with major ampullate (F-Ma) and acinous (F-Ac) glands. The ancestral mygalomorph (bird eating) spiders *Antrodiaetus unicolor* (Antrodiaetidae) with Aciniform glands (A) and *Aphonopelma chalcoldes* (Theraphosidae) with Acinous glands (T). Outgroup is the highly specialised, domesticated *Bombyx mori*, (Insecta: Bombicidae) with dope from its pair of large identical glands (bmx – silkworm silk). The grey area denotes the 95% regression confidence interval. (adapted from Dicko et al [25]).