Protein unfolding versus $\beta$-sheet separation in spider silk nanocrystals

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Abstract
In this communication a mechanism for spider silk strain hardening is proposed. Shear failure of $\beta$-sheet nanocrystals is the first failure mode that gives rise to the creation of smaller nanocrystals, which are of higher strength and stiffness. $\beta$-sheet unfolding requires more energy than nanocrystal separation in a shear mode of failure. As a result, unfolding occurs after the nanocrystals separate in shear. $\beta$-sheet unfolding yields a secondary strain hardening effect once the $\beta$-sheet conformation is geometrically stable and acts like a unidirectional fibre in a fibre reinforced composite. The mechanism suggested herein is based on molecular dynamics calculations of residual inter-$\beta$-sheet separation strengths against residual intra-$\beta$-sheet unfolding strengths.

Keywords: $\beta$-sheet, nanocrystals, polyalanine poly(Ala), polyglycine poly(Gly), protein structure
Classification numbers: 2.00, 2.01, 2.06, 5.10

1. Introduction
It is well established that spider silk is a hierarchically structured material with incredible properties of toughness [1–8]. These properties are not related to any one single aspect of silk, but rather exist as a combination of multi-level structural and physico-chemical characteristics. Silk is made up of basic amino acid units, which form both semi-amorphous and crystalline regions. The semi-amorphous regions are glycine rich and polyalanine (poly(Ala)) segments within these domains are normally less oriented than the poly(Ala) rich crystalline regions [9]. Such oriented regions form $\beta$-sheet nanocrystals, which are the load bearing reinforcements in silk and consist of ordered poly(Gly-Ala) [10, 11] and poly(Ala) repeat sequences [12]. The $\beta$-sheets are stacked [13] and interconnected by predominantly electrostatic forces of attraction [14] and thus bear the dual attributes of high strength and flexibility. Such proteins are able to dissipate considerable mechanical energy [15], which is evidenced in the enormous strength retention characteristics of silk fibres during elongation. Crystalline $\beta$-sheets typically constitute the lower fractional content in silk with values often quoted between 0.15 and 0.40 [7, 9, 13] for dry silk fibres. Spiders are able to vary this fraction of crystallization, which in turn alters the stiffness, strength and toughness of the dragline fibres [7, 16]. This crystalline fraction moreover deviates with reeling speed and species [17], as indeed does the size of the $\beta$-sheet nanocrystals [11].

Model studies of $\beta$-sheet nanocrystals have thus far brought to light the fact that these crystals have directional properties [5] and size effects as a function of crystallographic direction may enhance or diminish the mechanical properties [4, 5]. Moreover, there is an inherent reliance on the extent of poly(Ala) crystallinity relative to less orderly poly(Gly) repeats within silk fibroins [18], and protein units within these fibroins are generally oriented to the meridional direction [19] with the misaligned crystalline domains [9, 11, 20–22] realigning on deformation [23].

An important aspect of silk under investigation is that of strain hardening and the microstructural factors that give rise to this phenomenon. Failure and strain hardening within silks may arise through shear failure or combined flexional-shear failure in layered $\beta$-sheet crystals yielding smaller, higher toughness crystals [4], though $\beta$-sheet ‘fragmentation’ may also contribute to strain weakening [24]. The same authors [24] suggest that failure and strain hardening occurs via unfolding of the individual $\beta$-sheet proteins, which would...
increase resistance along the meridional direction of the silk under strain.

Silk is a biocompatible material with significant potential in man-made biomimetic materials [25–28], as well as in biocompatible biomaterials [10, 29–31]. In this communication, the GAGAAAAA sequence of Nephila pilipes [24] is modelled with an intention of elucidating the potential mechanisms of failure in β-sheet nanocrystals and how they may affect strain hardening in spider silk fibroins. To achieve this objective, molecular dynamics simulations are conducted. The β-sheet nanocrystals can have extended (a, b, c) (length, width and depth, respectively) crystalline dimensions. With an increased number of monomer units, the β-strands can be lengthened. Increasing the number of β-strands and turns will increase the width. Stacking the β-sheets in a laminated array will increase the crystallite depth. Finite element strength analyses in [5] made use of systematic arrays of solid cylinders to model crystallographic growth in three axes of orthogonal symmetry. Herein, molecular models are used to extend the crystal in each direction (a, b, c). As a result the conformational twisting of β-sheet structures that result from the (ϕ, ψ) angles in the molecule and their effects on strength can be included.

2. Methods

Abalone molecular dynamics software version 1.8.45 was used to assemble and optimize folded protein structures. Each structure was optimized to a state of lowest energy. A repeating amino acid chain sequence of GAGAAAAA was used to construct the individual β-sheets. This sequence is typical for the crystallite regions in the dragline silk of Nephila pilipes [24]. Five β-sheet β-strands (ϕ = −125°, ψ = 127°) were coupled with four-monomer II-turns and (ϕ, ψ) angles at (−141°, 89°), (76°, −130°), (−85°, −4°) and (−93°, 142°) to form β-sheets with antiparallel β-strands [22, 32]. An Amber94 force field was implemented into the simulations, applying also a dielectric permittivity constant for a glycinine rich environment [33]. In this force field, the bond and angle terms are modelled with harmonic potentials, the van der Waals (VDW) terms follow 6–12 potentials, the torsional terms are modelled with harmonic potentials, the van der Waals (VDW) terms follow 6–12 potentials, the torsional terms are modelled with harmonic potentials, the van der Waals (VDW) terms follow 6–12 potentials, the torsional terms are modelled with harmonic potentials.

The total molecular energy computed both within and between the β-sheets was converted to values for strength, σ_ult, according to equation

\[ σ_{ult} = (2EV^{-1})ε_{ult}^{-1} \]  

which is similar to the conversion used in [36], where total molecular energies were converted to the Young modulus equivalents.

To calculate the strength between the β-sheets, the same equation was used except the energy value, E, only included the total of the electrostatic and VDW energies between the sheets and the volume, V, was calculated for the space between the β-sheets. Similarly, to calculate the strengths of only the β-sheets, the total energy of the β-sheets were used alongside the sum total (a, b, c) crystal volume occupied by only the sheets. The ultimate strain, ε_ult, value here is 0.1 and is within a range of values inferred from Cetinkaya et al [5] and Giesa et al [37]. The underlying assumptions in equation (1) are that the β-sheet has the characteristics of a brittle material, which is evidenced in the simulations of Cetinkaya et al [5], and that all attractive energy, inclusive of both primary and secondary bonding, within the molecule contributes to the total strength output. At the molecular level, bonds are the primary reasons for structural integrity and resistance to material failure [38] and the reliance on bond energy for determining strength in this model is hence deemed suitable.

3. Results and discussion

3.1. Effects of β-sheet stacking on the specific strength of silk crystals

β-sheets with five antiparallel β-strands and four II-turns were constructed. Each sheet was made of 56 amino acid monomers with a GAGAAAAA repeat unit. The β-sheets were stacked by translating each sheet a distance of 10 Å similarly to [18], thereby creating the β-sheet nanocrystals. Single β-sheet simulations were primarily undertaken and β-sheet nanocrystals were then simulated using a further 2–11 β-sheets.

Figure 1 shows a graph of strength for stacked β-sheets and includes curves for the strengths calculated between the β-sheets, within the β-sheets and the strength calculations for the entire nanocrystal volumes. The total strength values of the entire β-sheet nanocrystals are indicative of the combined strengths of the β-sheets alongside the strengths that arise through intermolecular electrostatic and VDW forces. The total strength is essentially a disproportionately weighted average of both the intra- and inter-β-sheet strengths, and the range of strength values predicted here (0.7–2.6 GPa) are very close to the predictions made by Cetinkaya et al [39] where a value of 2.0 GPa was stated and by Cetinkaya et al [5] where a minimum to maximum range of 0.4–2.4 GPa is given. This gives credibility to the methods of energy to strength transformation used in this model. The intra-β-sheet strengths are the highest and this is expected due to the
high conformational energies of the folded poly(Gly-Ala) structures. These intra β-sheet strengths are also found to increase nonlinearly as a function of increased molecular mass and hence, nanocrystal volume. This is most likely due to the volumetric effects from the electrostatic forces of attraction between neighbouring β-sheets, which then effectively raise the intrinsic energies of attraction within the β-sheets. Provided the forces of attraction are higher than the repulsive forces, and the electron volumes overlap with electrostatic attractions dominating, the stability of the stacked β-sheets will be higher than they would be for individual β-sheets and the overall strength will rise. The inter-β-sheet strengths are considerably lower than the intra-β-sheet strengths but most interestingly, the total strength of the nanocrystals decreases as the number of stacked β-sheets increases after rising to an apical sheet stack configuration of three β-sheets. β-sheet nanocrystal strengths do decrease as a function of a lateral increase in size and stacking sequence [4, 5], however, herein it is discovered that an optimal stacking arrangement can exist, given the parameters used in the models.

### 3.2. Strength as a function of β-sheet nanocrystal length

In this section the effects of lengthening the β-strands are compared to the properties of strength. Three sets of β-sheets with five β-strands and four II-turns were constructed. In each case the β-sheets were stacked using translations of 10 Å [18] to create the β-sheet nanocrystals. β-sheets of length 27.68, 31.65, 34.56 and 39.79 Å were constructed using the same conformations as in the previous section, except that additional amino acid monomers were appended to each β-strand to lengthen or shorten the nanocrystal. The basic repeat unit remained as GAGAAAAA.

Shorter β-sheet nanocrystals have superior mechanical properties [4]. This also proves to be true for the inter-β-sheet strength as calculated herein (table 1). Extending the length of the β-strands increases the maximum achievable strength between the β-sheets and gives further weight therefore to the findings of Cetinkaya et al [5]. The maximum inter-sheet strength rises nonlinearly with respect to the length of the nanocrystal though. This is attributed to the conformational effects that arise from coupling the (ϕ, ψ) angles of the II-turns and β-strands, which create twisted and geometrically asymmetrical β-sheets. The result is that as the β-strands are extended, the electrostatic forces of attraction between the β-strands do not rise proportionally to the volume of the crystal unit cell. Table 1 provides values for the maximum inter-sheet strength for different length nano-crystals. It can be seen that the factor of difference for the inter-β-sheet to total strength is highest in the smallest nanocrystals. The difference progressively drops as the lengths of the β-strands are increased. The general tendency is for the inter-β-sheet strength to increase with respect to increasing β-strand length, while the total nanocrystal strength decreases (the only anomaly being for β-strand length 27.68 Å). Nevertheless, the highest overall strength is found to exist in the nanocrystals comprising eight-monomer β-strands (31.65 Å). It has been reported previously that smaller nanocrystals have higher strength and stiffness [4, 37], and that longer β-strands give rise to superior mechanical properties. An important additional concept is proposed here in conjunction with the findings of section 3.1, which is that there are certain optimal β-strand lengths as well as certain optimal β-sheet stacks. In the case here, the 27.68 Å β-sheets have strands made up of four amino acid monomers. Given that the II-turns are also four-monomers long, rather than that nanocrystals made up of these β-sheets exhibit directional characteristics, the structure of the β-sheets resemble more of an aspect ratio 1:1 ‘block’ and hence the strength is low. When the number of monomers making up the β-sheet β-strands rises to eight (31.65 Å), the β-sheet exhibits the directional properties reported in the literature [4, 5] and these nanocrystals fall into the category of smaller nanocrystals with the highest strength and stiffness properties in particular directions. As the number of monomers is increased further per β-sheet β-strand, the strength properties of the nanocrystals drop. This is because the conformational structure of the longer β-sheets occupy significantly greater spatial volume, solely as a function of the II-turn couplings to the β-strand and the existing (ϕ, ψ) angles.

### 3.3. A proposed mechanism of nanocrystal failure and its subsequent influence on the strain hardening of spider silks

In this section residual strength is compared for an unfolding β-sheet against two β-sheets separating laterally in a shearing mode of failure. These are both failure mechanisms that have been proposed in the literature [4, 24] though there are no thorough studies comparing and contrasting the two in relation to each other and the overall mechanical properties of silk. For the simulations, two β-sheets are stacked on top of each other, each sheet containing 56 monomers in a repeat GAGAAAAA sequence with an eight-monomer β-sheet to four-monomer II-turn conformation and (ϕ, ψ) angles as described earlier. In β-sheet separation failure, the β-sheets are effectively moved at increments in a shear mode of failure and molecular energies calculated at each displacement. For the β-sheet unfolding, the sheet is essentially ‘pulled open’ from the disconnected ends four-monomer units at a time, two from each end, and calculating the molecular energies at each step.

Figure 2 shows the residual strength plotted against the total deformation prescribed to the nanocrystal. Although two β-sheets were stacked and modelled for this portion of the research, the strength values for only one of the unfolding β-sheet proteins is plotted. The two curves in figure 2 are distinctly dissimilar. The unfolding β-sheet has considerably higher residual strength on unfolding than can be found between the β-sheets at any given level of

| β-strand length (Å) | Inter-β-sheet strength (GPa) | Total nanocrystal strength (GPa) | Factor of difference | Cross section [b · c] (Å²) |
|---------------------|-----------------------------|-------------------------------|---------------------|--------------------------|
| 27.68               | 0.229                       | 1.67                          | 7.29                | 2146                     |
| 31.65               | 0.348                       | 2.51                          | 7.21                | 5765                     |
| 34.56               | 0.362                       | 2.02                          | 5.58                | 6775                     |
| 39.79               | 0.418                       | 1.85                          | 4.43                | 8658                     |

Table 1. Strength values for different length nanocrystals.

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deformation and displacement. Remembering that the residual strength between the β-sheets is calculated solely from the combined electrostatic and VDW forces that exist between them, this comes as no surprise. However, unlike the case of shearing β-sheets where the forces of attraction between the two sheets progressively approaches zero as separation increases, the residual strength of the unfolded β-sheet falls and then rises to yield, at the highest level of deformation, a residual strength value higher than that of the original β-sheet. Strength is an inversely proportional function of the cross sectional dimensions. As the β-sheet unfolds, the angulated structure is not sturdy, is less bound by electrostatic forces and consequently the potential energy of the system is spread over a very large area in space. As the protein continues unfolding into a single strand, the area over which the potential energy is distributed decreases and hence the strength of the β-strand rises. As the protein molecule approaches the conformation of a single β-strand, the strong covalently bonded backbone of C–C (346 kJ mol$^{-1}$) and C–N (305 kJ mol$^{-1}$) bonds provide the resistance to deformation over a significantly smaller \$b \cdot c\$ crystallographic area.

Given these findings, it is herein hypothesized that there is a specific step-by-step mechanism by which strain hardening occurs in spider silk. Figure 3 shows a pictorial schematic of this mechanism. In this figure elongation of the silk fibroin leads to shearing in the β-sheet nanocrystal. The first mechanism of failure is the separation of β-sheets from each other, creating smaller β-sheet nanocrystals (or individual β-sheets). This has been deemed an important mechanism for strain hardening in [4] and contrarily, to strain weakening in [24]. The research presented here indicates that both cases are possible and will ultimately depend on whether the stress resistance prior to failure in the original nanocrystal is larger or smaller than the combination of stresses resisted in the fragmented crystals (refer to the curve for total crystal strength in figure 1). The smaller nanocrystals (or individual β-sheets) can result in strain hardening within the silk fibroin on the condition that the newly created smaller nanocrystal fragments resist higher loads than the original crystal prior to failure. The extent of mechanical resistance will depend on the length of the β-strands coupled to the extent of stacking of the newly created nanocrystals.

Figure 4 summarizes the effects of changing the β-strand length and the magnitude of β-sheet stacking with respect to their effects on the total nanocrystal strength. The strongest nanocrystals are represented by the solid black circle and are the intersection of two maxima. Deviation from this intersection results in weaker nanocrystal structures. The weakest nanocrystals have the smallest β-strands such that the β-sheet is more like a 1 : 1 aspect ratio ‘block’. Interestingly, the structural sizes and conformations of the highest and lowest strength nanocrystals are not largely dissimilar and are proximally close on this strength-size map (figure 4). The creation of smaller nanocrystals, if sufficiently stiff and strong, contributes to stage I of strain hardening in the fibroin. Smaller nanocrystals, in turn, give rise to heightened localized strain energy densities. Therefore, unfolding the β-sheets may actually occur relatively soon after the nanocrystals separate into smaller segments of stacked or individual β-sheets. The point at which unfolded β-sheets also contribute to strain hardening will be the point at which they start becoming more ‘strand-like’ and have a more stable geometrical configuration (refer to figure 2). This is proposed to be, therefore, stage II of strain hardening. Molecular chain alignment in the semi-amorphous regions giving rise to strain stiffening has already been reported in [38]. The reinforcing effect
of an unfolded β-sheet is expected to act similarly to that of a reinforcing fibre in a unidirectional fibre reinforced composite.

4. Summary

Based on numerical calculations, herein it is suggested that strain hardening in spider silk occurs primarily through the separation of β-sheets whilst protein unfolding is suggested to be a potential secondary source of strain hardening.

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Figure 4. Summary chart on the structure–strength relationships of β-sheet nanocrystals.
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