Pseudoelastic behaviour of a natural material is achieved via reversible changes in protein backbone conformation

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The egg capsules of marine prosobranch gastropods, commonly known as whelks, function as a protective encapsulant for whelk embryos in wave-swept marine environments. The proteinaceous sheets comprising the wall of whelk egg capsules (WEC) exhibit long-range reversible extensibility with a hysteresis of up to 50 per cent, previously suggested to result from reversible changes in the structure of the constituent protein building blocks. Here, we further investigate the structural changes of the WEC biopolymer at various hierarchical levels using several different time-resolved in situ approaches. We find strong evidence in these biological polymers for a strain-induced reversible transition from an ordered conformational phase to a largely disordered one that leads to the characteristic reversible hysteretic behaviour, which is reminiscent of the pseudoelastic behaviour in some metallic alloys. On the basis of these results, we generate a simple numerical model incorporating a worm-like chain equation to explain the phase transition behaviour of the WEC at the molecular level.

Keywords: pseudoelasticity; biopolymer; protein; coiled-coil; conformation

1. INTRODUCTION

Choosing suitable materials for packaging or encapsulation of valuable goods involves a number of considerations, such as the mechanical robustness, susceptibility to degradation and environmental sustainability of the material. Similarly, for biological organisms, the logistics of reproduction typically necessitate a large energy investment in materials and strategies for safeguarding a developing embryo. For example, oviparous organisms (i.e. those which lay eggs) fabricate material barriers such as shells, gel masses and capsules to guard against mechanical perturbation and microbial degradation, while simultaneously allowing for gas exchange and selective permeability to small molecules [1]. The adaptive value of such materials reflects the evolutionary selective pressures inherent in the environment, and therefore, in extreme habitats such as the wave-swept marine intertidal zone, it is not surprising that the mechanical behaviour tends towards increased toughness and damage tolerance.

The subject of the current study, the marine whelk Busycotypus canaliculatus, lays its encapsulated eggs in the shallow waters of the North Atlantic where, during maturation, they are exposed to intense hydrodynamic forces, hydration extremes, microbial attack and predation (figure 1a) [2]. Along these lines, the biopolymeric material that composes the whelk egg capsule (WEC) dissipates up to 50 per cent of applied mechanical energy via hysteresis during cyclic loading; however, it recovers initial mechanical properties instantaneously when brought back to its initial length (figure 1b) [3]. As pointed out previously, this behaviour is reminiscent of pseudoelastic (sometimes called superelastic) alloys such as those based on Ni–Ti [4,5]. In contrast to the shape-memory effect observed in certain alloys and polymers [6], pseudoelastic behaviour does not require an additional non-mechanical trigger to induce the change between the high-strain and low-strain phases. Additionally, pseudoelastic materials typically exhibit a large hysteresis between the loading and unloading curves. As suggested by previous mechanical models of the WEC, the observed reversible conformational change of the constituent proteins is analogous to the phase transition between the low-strain phase and high-strain phase of a pseudoelastic alloy. The similarity in mechanical behaviour points to a possible common physical
underpinning [4,5]; however, this requires a stronger under-
standing of the molecular-level behaviour of the WEC.

The WEC biopolymer is one of many examples of
notable biological load-bearing structures such as hair
keratin, silk, tendon and mussel byssal threads that are
constructed almost entirely from protein building
blocks. The mechanical behaviour of protein-based
load-bearing materials is intimately linked to the confor-
mation of the constituent polypeptide building blocks,
which typically consists of stable secondary structures
and limited tertiary structure. For example, many species
of cartilaginous and jawless fishes also produce egg
 capsules, which in some well-characterized examples are
composed of a highly sclerotized collagen-based material
that imparts high strength and toughness [7]. The WEC,
on the other hand, is believed to be composed primarily
of proteins in an α-helical coiled-coil secondary struc-
ture [5]. While some systems use coiled-coils to create
materials nearly as stiff as collagens, the coiled-coil is
considerably more compliant than the collagen triple
helix at the molecular level [8].

The α-helical coiled-coil is a ubiquitous conformational
motif in proteins used throughout nature in which two
to seven peptide chains in a right-handed α-helical con-
formation intertwine to form a left-handed super-helix
that is more energetically stable than an individual
α-keratins, myosin and fibrinogen. These proteins in
turn compose load-bearing materials with a wide array of
functions and mechanical properties, such as extra-
cellular matrix intermediate filaments, hair and wool
fibres, blood clots and hagfish slime [9–13]. α-Helical
coiled-coils possess unique attributes as load-bearing
elements owing to their compact structure (rise/residue
in the helical structure = 1.5 nm), their ability to
extend to large deformations reversibly (up to 2.5 times
the folded length) [12,13] and their hydrogen-bonding
network, which runs along the helical axis. These factors
are believed to contribute to the extensibility and stiff-
ness of hair keratin, fibrin clots and hagfish slime
threads [10,11,14].

Amino acid sequence variations within individual helices
can lead to structural and topological differences
between the coils of different proteins. Single molecule
force spectroscopy experiments on myosin and fibrino-
gen coiled-coils have revealed that such differences
strongly influence mechanics of unfolding and refolding
[12,13]. In fact, researchers are currently using protein
engineering in order to tune the mechanical behaviour
of recombinant coiled-coil proteins [15]. Variation in
mechanical behaviour of materials based on coiled-
coils can additionally arise from differences in tissue
processing and the presence of accessory proteins [8].
Therefore, although the molecular-level mechanical be-
haviour of coiled-coils is now better understood, the
structure–property relationships that define the mech-
anical behaviour of bulk materials comprised from
coiled-coils are still somewhat unclear. In order to
reconcile the large diversity of mechanical behaviour
in bulk materials based on coiled-coils, the structure–
function relationships need to be understood for each
individual material at the molecular level up through
higher levels of structural hierarchy.

Previously, the underlying structure of the WEC
biopolymer was studied with traditional X-ray diffraction
on static samples, suggesting that the mechanics are
governed by changes in protein secondary structure
[5]. Here, we further investigate the structural changes
during mechanical deformation of WEC from the
species B. canaliculatus using three measurement tech-
niques that each provide time-resolved in situ structural
information about the material at different levels of
hierarchy. In situ confocal Raman spectroscopy provides
molecular-level information about the conformation
and orientation of backbone atoms in the protein.
Time-resolved synchrotron wide angle X-ray diffraction
(WAXD) provides information about ordered secondary

Figure 1. WEC mechanical performance. (a) Strand of egg capsules laid by B. canaliculatus (illustration top-right inset). For mechanical testing, individual egg capsules were removed from the strand, and strips were excised as indicated in the bottom-right inset. (b) Typical tensile stress–true strain curve for a strip of WEC material cycled four times with no rest between cycles. Arrows indicate loading and unloading, and the yield region of the curve is highlighted in grey.

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structures that exist in the material as well as their orientation while the material is being deformed. Small angle X-ray scattering (SAXS), also performed with synchrotron radiation, provides information about larger order hierarchical structures with repeating electron density profiles. Observations at each level of hierarchy support the existence of two distinct structural phases. The low-strain phase in the elastic region prior to yielding (white region at low strain in figure 1b) corresponds to an α-helical conformation of the protein subunits. The high-strain phase after yielding is related to an extended and axially unstructured conformation of the proteins, which we call β*-phase. It was observed that the two phases coexist during yield (grey region in figure 1b) without the appearance of an intermediate conformation. This is consistent with predictions made by the previously proposed molecular-level models [4,5]. However, it was also observed that the low-strain and high-strain phases behave elastically. This investigation indicates that the molecular-level mechanical behaviour of the coiled-coil load-bearing elements in the WEC behave quite differently from the archetype α-helical to β-sheet transitions observed in materials such as hair keratin and hagfish slime threads [10,11], which may underlie the stark differences in macroscopic mechanical behaviour. We present a modified mechanical model that incorporates these new observations and that offers an explanation for the mechanical similarity of the WEC to pseudoelastic metal alloys.

2. EXPERIMENTAL PROCEDURE

2.1. Sample preparation

Buscotypus canaliculatus egg capsules were purchased from the Marine Biological Laboratories (Woods Hole, MA, USA). Capsules were cut with a razor into approximately 5 x 10 mm strips and washed repeatedly in distilled (DI) water using gentle physical disruption to remove the mucous lining. Prior to testing, samples were stored in DI water for up to several weeks. The exact thickness and width of each strip were measured with a micrometer.

2.2. X-ray scattering

A custom-made, portable, uniaxial tensile testing apparatus called the Micro-mechanical Tensile Apparatus (MiTA) was used for stress–strain measurements during X-ray scattering [16]. The device was placed so that the samples were directly in the beamline during loading and unloading, allowing real-time in situ tensile testing. Water vapour was directed onto samples by a nebulizer to ensure complete hydration throughout the tensile cycle. A strain rate of 8 mm s⁻¹ was used for both extension and relaxation and grip separation distance of the device calipers was used for sample length. All results are given in engineering stress versus true strain.

X-ray data collection was performed on the MuSpot beamline at the BESSY synchrotron source (Berlin Elektronenspeicherring Gesellschaft mbH, Berlin, Germany) [17] with an X-ray wavelength of 1.0 Å and a beam size of 30 μm. Beamline calibration was carried out with a corundum (Al₂O₃) standard, giving a sample to detector distance of 256.39 mm for WAXD and 906.39 mm for SAXS measurements. A two-dimensional CCD detector (MarMosaic 225, Mar USA, Evanston, USA) with pixel size of 73.24 μm and resolution of 3072 x 3072 pixels was used to acquire the frames. Samples were exposed to X-rays for 30 s durations, while the MiTA was actively recording tensile data. As the strain rate was so low, the engineering strain of all samples changed by no more than 2 per cent during exposure times. Analysis was performed using the Fit2D program [18]. Intensity around the meridian and equator was integrated radially using the CAKE command, and the shift and change in peaks were tracked as a function of externally applied stress and strain.

2.3. Raman spectroscopy

For Raman micro-spectroscopy, a continuous laser beam was focused down to a micrometre-sized spot on the sample through a confocal Raman microscope (CRM200, WITec, Ulm, Germany) equipped with a piezo-scanner (P-500, Physik Instrumente, Karlsruhe, Germany). The diode-pumped 785 nm near infrared (NIR) laser excitation (Toptica Photonics AG, Garching, Germany) was used in combination with a water immersed 60x (Nikon, NA = 1.0) microscope objective. The linearly polarized laser light was rotated using a half-wave plate, and scattered light was filtered by introducing a further polarizer (analyser) before the confocal microscope pinhole. The spectra were acquired using a CCD (PI-MAX, Princeton Instruments Inc., Trenton, NJ, USA) behind a grating (300 g mm⁻¹) spectrograph (Acton, Princeton Instruments Inc., Trenton, NJ, USA) with a spectral resolution of approximately 6 cm⁻¹. ScanCtrlSpectroscopyPLUS (v. 1.38, WITec, Ulm, Germany) and WITecProjectPLUS (v. 2.02, WITec, Ulm, Germany) were used for the experimental setup and spectral data processing, respectively. Chemical images were achieved by integration over defined Raman shift regions in the spectrum using a sum filter. The filter calculates the intensities within the chosen borders, and the background is subtracted by taking the baseline from the first to the second border. The amide III intensity was obtained by integrating the intensity of bands in two spectral regions corresponding to α (1195–1255 cm⁻¹) and β* (1270–1350 cm⁻¹) conformations. The tissue was stretched at a constant rate of 20 μm s⁻¹ to fixed strain values and allowed to relax for approximately 5 min before measuring. On the basis of the similarities between the behaviour of α to β* conversion in WAXD and Raman (figure 5), we believe that the effect of relaxation does not greatly influence our interpretation of the results. At each strain value, the sample was scanned to a depth of 50 μm with steps of 1 μm (in each point the signal was integrated for 1 s) and five scans for each depth level (measuring deeper than 50 μm was not possible owing to a drop off in signal). Four depth measurements (with analyzer and analyser parallel, perpendicular and cross polarized with respect to the strain direction) were performed and isotropic (polarization independent) spectra were calculated using a procedure as described previously.
3. RESULTS AND DISCUSSION

3.1. Mechanical characterization

The stress–strain data acquired during in situ tensile testing did not vary between individual samples and are in agreement with previously published findings on the material [2,5,20]. An example of a stress versus true strain curve can be seen in figure 1b. As we are most interested in the force per molecule $f_p$ within the proteinaceous tissue, we need a quantity proportional to it. Hence, throughout the paper, we use $\sigma = f_p N/A_0$, where $N$ is the number of loaded molecules in the initial cross section with area $A_0$. It so happens that $\sigma$ is equal to the engineering stress, but most importantly it is proportional to $f_p$ just by a constant factor. The curve shows an initial region of high modulus from 0–5% true strain. At this point, the material yields at nearly constant stress (grey area in figure 1b). At approximately 40% per cent true strain, the material once again stiffens, and this increased modulus is maintained up to 69 per cent true strain. Upon unloading, there is a large hysteresis loop (approx. 50%) observed as the material relaxes back to its original length. As shown previously [2], no permanent damage is inflicted during yield—further cycles are practically identical to the first.

3.2. In situ wide angle X-ray diffraction

Strips of egg capsule were stretched continuously in the hydrated state at a low extension rate, while WAXD measurements were made continuously with synchrotron radiation at predetermined strain values in order to acquire time-resolved information about structural transitions in the backbone of the load-bearing protein components. As observed in previous static WAXD measurements on WEC material [5], unloaded strips of capsule wall material exhibit peaks with a $D$-spacing of approximately $0.53 \text{ nm}$ along both the meridian and equator (figure 2a). These peaks correspond to the axial periodicity along coiled-coil $\alpha$-helices [11] and suggest that this material has $\alpha$-helical fibres oriented orthogonally as previously observed [2,5,20]. On straining the capsule material uniaxially from 0 to 5 per cent, true strain does not change these diffraction patterns appreciably. When the material is extended beyond the yield point, the intensity of the meridional peaks at $0.53 \text{ nm}$ corresponding to the $\alpha$-helices aligned along the axis of tenacity decreases and an equatorial peak with a $D$-spacing of approximately $0.47 \text{ nm}$ appears (figure 2b). This equatorial reflection is reminiscent of the lateral spacing between protein chains in a $\beta$-sheet conformation; however, we do not observe the characteristic axial peak at approximately $0.33 \text{ nm}$, which corresponds to the distance between residues (rise/residue) along the $\beta$-strand protein backbone and which is diagnostic for the existence of a $\beta$-sheet phase [11]. This is in stark contrast to stretched hair keratin or hagfish slime threads in which these axial reflections are clearly observed in stretched tissue samples [10,11]. Miserez et al. [5] did observe a faint axial reflection in dried WEC samples after reaching approximately 80 per cent true strain; however, consistent with our measurements, they were never observed in hydrated samples at similar strains. Results on hydrated tissue are most relevant because WEC is a marine material. Thus, we are forced to conclude that a true $\beta$-sheet conformation is not present in stretched WEC and, therefore, refer to the secondary structure in the stretched WEC biopolymer simply as extended conformation ($\beta^*$). The transitions in the intensity of the $\alpha$ and $\beta^*$ peaks that occur between resting state and post-yield stiffening will be discussed in more detail later.

3.3. In situ small angle X-ray scattering

SAXS exposures of WEC in the resting state produce visible reflections up to order $n = 11$, and are identical along both the equator and meridian, further supporting the existence of orthogonally oriented fibres (figure 3a,c). The calculated fibril radius is $9 \text{ nm}$ with $D$-space values of approximately $100 \text{ nm}$ running along them. When the material is put under tension, changes are observed in the SAXS reflections along the meridian, whereas the equator is less affected. When stretched further into the yield region (more than 30% true strain), a second set of reflections, representing a different structural phase, dominates along the meridian (figure 3b,c). Notably, both phases are observed simultaneously in certain measurements made at approximately 30 per cent true strain, confirming that they in fact represent separate phases in which the proteins are in two different conformational states. Although the structural origin of the $100 \text{ nm}$ periodicity in unstretched WEC tissue is unclear, we refer to the two structural phases as $\alpha$ and $\beta^*$, respectively, because the transition between them occurs at similar strain values as the $\alpha$ to $\beta^*$ transition observed in WAXD. The details of the transition between these phases will be discussed in more detail later.

3.4. In situ confocal Raman spectroscopy

Confocal Raman spectroscopy is a useful technique for studying protein secondary structure in biological
materials in situ due to the sensitivity of several bands (amide I and amide III) to the specific conformation of the peptide backbone [21]. For each amino acid residue in a peptide backbone, the conformation is determined by the dihedral angles $\Phi$ and $\Psi$, which describe the torsional rotation about the two bonds attached to each $C_a$ atom (figure 4a). The dihedral angles corresponding to $\alpha$-helical and extended conformations are confined to a limited range of values, each representing minima in the conformational energy landscape (figure 4b) [22].

Typical isotropic Raman spectra obtained from an unstrained WEC sample and the same sample at various strain values can be seen in figure 4c. The positions of the amide I and amide III bands are consistent with an $\alpha$-helical protein conformation in the unstretched state, corroborating the WAXD results [21,23,24]. Stretching of the tissue results in a shift of the amide I band to higher wavenumbers, the development of a separate peak at a lower wavenumber within the amide III band and the emergence of a peak at approximately 1400 cm$^{-1}$ due to $C_\alpha$–$H$ stretching. Changes in the amide III band are particularly informative for monitoring changes in backbone secondary structure in protein structural studies [25,26]. On the basis of previous studies with model peptides of known conformation, the position of the amide III band in Raman spectroscopy primarily depends on the $C_\alpha$ dihedral angle in the protein backbone such that compact $\alpha$-helical conformations produce vibrations in the range of 1270–1350 cm$^{-1}$ and more extended conformations seen in $\beta$-strands, random coil, polyproline II and polyglycine II produce lower energy vibrations in the range of 1195–1255 cm$^{-1}$ (figure 4b) [25].

While the intensity values for these two peaks are not quantitative, the changes observed in the amide III band of WEC during stretching, as well as the changes in the amide I and $C_\alpha$–$H$ vibrational bands, clearly indicate a transition from $\alpha$-helical to more extended ($\beta^\alpha$) protein...
backbone conformations (figure 4c) [21,23,26]. The valley between the two peaks in the amide III band indicates a paucity of intermediate protein conformations with $\Psi$ dihedral angles corresponding to the partially restricted and energetically unfavourable conformations situated between $\alpha$-helical and extended conformation, known in the literature as the ‘bridge’ (figure 4b,c) [22,25]. These results suggest that the transition of individual $\alpha$-helices to the extended conformation occurs rather suddenly as a ‘jump’ over the bridge region by primarily increasing the $\Psi$ angle of constituent residues. On the other hand, at the tissue level, the change occurs progressively and discontinuously because not all the $\alpha$-phase vanishes at once. This suggests a progressive

Figure 4. In situ confocal Raman spectroscopic characterization of protein conformation in WEC. (a) Visual models of the peptide backbones in an $\alpha$-helical versus $\beta$-strand conformation. A detailed structure of the $\beta$-strand conformation is shown, including side chains, to clarify the position of the dihedral bonds. (b) Rough illustration of a typical Ramachandran plot in which the $\Phi$ and $\Psi$ dihedral angles are plotted on opposite axes. The dihedral angle combinations representative of $\alpha$-helical (green) and extended $\beta^*$ (red) are outlined. The blue region represents partially allowed, but less energetically favourable conformations. White regions represent conformations that are not allowed for the majority of amino acid residues owing largely to steric conflicts. (c) Isotropic Raman spectra from the WEC tissue at several different strain values. Prominent Raman bands (amides I and III) undergo transitions during strain that are consistent with a shift from $\alpha$-helical to more extended conformations ($\beta^*$). (d) Raman depth scan of WEC layers at rest and at 69% true strain. Intensity profiles from polarized Raman imaging represent the ratio between the area under the $\beta^*$ peak and $\alpha$ peak of the amide III band. Conversion from $\alpha$ to $\beta^*$ occurs only in layers where protein fibres are oriented in the direction of tension.
rupturing of $\alpha$ domains to $\beta^*$ conformation as discrete events during yield similar to the sacrificial domains in the muscle protein titin [27].

Polarization-dependent variations in the intensity of the amide I band of polarized Raman depth scans through the top 50 $\mu$m of the capsule wall occur in
10–15 μm thick layers and arise from differences in how the α-helices are oriented. This observed layering of fibres oriented 90° from each other is consistent with both the WAXD and SAXS data as well as with previous studies [2,5,20]. Notably, in Raman depth scans of stretched WEC tissue, the transition from a coiled α-helix to extended conformation occurs strictly in the α-helical protein fibres oriented in the direction of stretching (figure 4d).

3.5. Phase transitions during in situ cyclic tensile deformation

Figure 5 provides a summary of the structural transitions occurring in WEC strips at various hierarchical levels during stretching as measured by in situ Raman spectroscopy, WAXD and SAXS, respectively. The two columns of graphs present the same data points plotted versus relative stress (defined in the caption of figure 5) and true strain, respectively. Although the three measurement techniques are probing very different levels of hierarchy, the plots reveal many similarities. During the initially stiff region of the stress–strain curve up to 5 per cent true strain, there is no appreciable change in the conformation as seen in Raman and WAXD. Within the yield region, however, we observe a decrease in the amount of α-phase and an increase in the β*-phase in all three techniques. The discontinuous nature of the conversion between the two during yield is especially clear in the SAXS data. While the SAXS results must be addressed cautiously because the molecular source of the reflections is as yet unclear, the molecular true strain calculated based on the increase in D-spacing during deformation is linear and proportional to tissue true strain, with a slope of nearly 1. Considering the extensive protein unfolding indicated by Raman and WAXD measurements during yield, this strongly suggests that tissue strain is controlled almost entirely by the molecular strain of the protein chains aligned in the direction of stretching, rather than sliding of nanoscale domains as observed in other hierarchically structured materials such as tendon collagen. The relative stiffness of the α-phase was shown to be more than 20-fold higher than that of the β*-phase based on the molecular strain extracted from the SAXS data. This further suggests that the β*-phase is unlikely to consist of a crystalline β-sheet conformation or any other rigid secondary structure, which would be much stiffer. Furthermore, the SAXS data clearly show that the phase appearing at large strains (β*) is able to extend from a D-spacing of 140 nm to a D-spacing of 190 nm (as the WEC tissue goes from an overall true strain of approx. 35 to 70%). A crystalline β-sheet (0.33 nm rise/residue) could not be extended as much without breaking (the maximum allowable value is reported between 0.36 and 0.38 nm rise/residue [12,28]). The actual conformation of the β*-phase will be addressed further in the discussion.

During relaxation, there is a complete recovery back to the α-phase; however, the transition occurs at a lower relative stress producing a clear hysteresis at all three levels of hierarchy. Notably, the hysteresis is confined to the yield region, where there is a mixture of the α- and β*-phase. The absence of hysteresis in the single-phase regions indicates that the behaviour of the pure α- and β*-phases is essentially elastic. In the right-hand column of graphs (true strain), the hysteresis observed in the curves versus relative stress is largely missing, and the stretching and return curves are overlapped. This indicates that for a given strain value, there is a defined ratio of the two phases and that conformation is strain-dependent. Tensile mechanical hysteresis in engineering materials is connected to energy dissipation and may be attributed to internal friction or more generally the breaking of bonds [29,30]. While additional work will need to be done regarding potential interfibrillar slippage and friction associated with this system, it is reasonable to believe that a large part of the WEC hysteresis originates at the level of reversible protein conformational changes involving the breaking of hydrogen bonds.

Single molecule force studies on unfolding and refolding of myosin α-helical coiled-coils show a high elasticity with almost no hysteresis on time scales of less than one second [12], whereas experiments with the more topologically complex triple helical coiled-coils of fibrinogen showed a hysteretic behaviour more akin to the shock absorbing muscle protein titin, and was consistent with a worm-like chain (WLC) model [13]. The authors attributed the different behaviours between myosin and fibrinogen to the relative complexity of the coiled domains, which directly influenced the refolding rate of the protein. For example, the myosin double helical coiled-coil is uninterrupted for a length of 150 nm, whereas the fibrinogen coils are segregated into shorter segments of approximately 17 nm owing to covalent cross-links within the coil [12,13].

Only on the basis of protein mechanics, one might expect a greater superficial resemblance of WEC proteins to fibrinogen than to myosin and a more complex topological structure. While the sequences of the proteins composing the WEC remain unpublished at this time, they are currently available in the protein database (UniProtKB accession D2KC11, D2KC14, D2KC13, D2KC14). Initial analysis on the available sequence reveals multi-coiling in the WEC proteins with several interruptions that punctuate predicted α-helical domains, suggesting a superficial similarity to fibrinogen; however, further in-depth studies must be performed. One implication of the tentative similarity of the WEC proteins to fibrinogen is that the molecular-level deformation behaviour of the β*-phase under tension should follow a WLC model. This implication will be examined in more detail further on.

Other materials such as hair keratin and hagfish slime were previously determined to exhibit a mechanically induced α-helix to β-sheet transition, though, as mentioned, both materials are structurally and mechanically very different from the WEC and do not show nearly the same degree of reversibility that WEC tissue exhibits [10,11]. In these other materials, it was proposed that when coiled-coil α-helices are subjected to a load along the coil axis, the hydrogen bonds that stabilize the helices rupture and the tightly packed conformation gradually unravels, passing continuously through an...
3.6. Model for the $\alpha$- and $\beta^*$-phases

On the basis of our current observations, we propose a revised model in which a modular array of protein chain domains dominated by an $\alpha$-helical conformation exists in the unstretched state (figure 6a). As in hair keratin and the single molecule behaviour of myosin and fibrinogen, the initial modulus observed prior to yield results from the hydrogen-bonding network along the coiled-coil $\alpha$-helical backbone resisting deformation [11]. This is consistent with the elastic behaviour observed prior to yield in Raman and WAXD. At 5 per cent true strain, the same point at which coiled-coil intermediate filaments begin to unfold, the $\alpha$-helical structure begins to rupture in a modular fashion in which discrete domains unravel independently from one another, similar to the globular domains of the muscle protein titin [31]. The mechanics of this sort of modular unravelling have been examined in previous studies, and the resulting effect for bulk materials is a post-yield plateau wherein the apparent modulus does not increase much with the increase in strain [32]. When the $\alpha$-helical modules unfold due to rupturing of intermolecular hydrogen bonding parallel to the chain axis, they immediately transform to an unordered extended conformation ($\beta^*$) where adjacent chains assume a lateral spacing of 0.47 nm typical of a $\beta$-sheet, but without the axial order or large rise/residue typical of such a structure. The transition region around 40 per cent true strain represents the point at which a critical amount of $\alpha$-helical domains have been converted to $\beta^*$ producing a new SAXS pattern at

Figure 6. Model for the phase coexistence in WEC fibres. (a) Simple schematic of conformational changes occurring in the WEC biopolymer during mechanical deformation. Unfolding of $\alpha$-helical domains into a worm-like chain (WLC) extended conformation ($\beta^*$) begins occurring at the onset of yield (position C) as a sudden conversion of discrete domains. During yield (position D) more and more $\alpha$-domains are unfolded until all domains are in the WLC phase (position E). With further straining, the soft $\beta^*$ (WLC) domains continue to extend (position F). (b) Elastic energy of extended $\alpha$-helices (green) and WLCs (red) according to equations (3.1) and (3.2) and with the parameters given in the text. The black dotted line corresponds to the common tangent between the energy curves. (c) The stress in the fibre can be obtained by taking the derivative of the elastic energy density $\partial W/\partial s$ with slope $\sigma_Y$ producing a new SAXS pattern at

amorphous phase as it elongates and eventually forms new hydrogen bonds between adjacent protein chains, forming a crystalline $\beta$-sheet structure as evidenced by the distinct axial reflection observed in WAXD measurements. On the basis of similarities observed in WAXD on dried WEC samples, Miserez et al. [5] suggested a similar model in which changes in protein conformation ($\alpha$-helix to $\beta$-sheet) dictate mechanical behaviour and concluded that the elasticity of the WEC originated from primarily non-entropic sources. Our time-resolved in situ measurements on hydrated WEC tissue are consistent with several important aspects of the previously proposed model; however, the results additionally suggest that in the high-strain region, the reversible extensibility arises from the entropic behaviour of a largely disordered protein conformation ($\beta^*$) that exhibits elastic deformation behaviour, rather than an ordered $\beta$-sheet conformation. This difference may contribute to the clear pseudoelastic behaviour of the WEC vis-à-vis the other tissues.

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a larger D-spacing at approximately 145 nm. During the yield region, the converted β* domains continue to extend gradually while the remaining α domains unfold. In the high-strain region, most of the α domains have been unfolded and a further elongation of the tissue requires the stretching of the β* domains as evidenced by the continuous increase in the D-spacing.

For a fibrous material with protein chains running parallel to the stress direction, each of the protein chains feels a force \( f_0 \) that relates to the applied load \( F \) in the following way: \( F = Nf_0 \), where \( N \) is the number of loaded molecules in the initial tissue cross section \( A_0 \). Given that \( N \) is not changing during extension (as long as none of the molecules fractures), the applied load \( F \) remains directly proportional to the force per molecule \( f_0 \). The 20-fold lower stiffness of the β*-phase relative to the α-phase implies a non-crystalline extended conformation, which is consistent with the mechanical behaviour of parallel WLCs [33]. We therefore propose a very simple mathematical model to describe this behaviour. We assume that the fibres consist of a series of domains (figure 6a) with molecules that may be in an α-helical conformation (stabilized by intramolecular hydrogen bonding) or in an extended conformation (β*). The mechanical behaviour of extended proteins under tensile load is often described by the WLC model [33,34]:

\[
\begin{align*}
    f_0 &= \frac{\sigma A_0}{N} = \frac{kT}{l_p^\alpha} \left( \frac{1}{4} \left( 1 - \frac{s}{L} \right)^2 - \frac{1}{4} \frac{s}{L} \right),
\end{align*}
\]

where \( l_p^\alpha \) is the persistence length of the molecule, \( L \) is the length of the molecule in the fully stretched state (contour length) and \( s \) the actual length of the molecule effectively shortened by thermal bending fluctuations of the chain. If the molecule is present as an α-helix, its mechanical behaviour is assumed to be elastic

\[
\begin{align*}
    f_0 &= \frac{\sigma A_0}{N} = K(s - s_0),
\end{align*}
\]

where \( s_0 \) is the length of the α-helical molecule at rest and \( K \) is the spring constant describing the elastic behaviour of the α-helix. According to a standard thermodynamic treatment, the equilibrium length \( x \) of the molecule for a change from \( s \) to \( x \) follows from the minimum of the total (potential) energy (per unit area), \( W(x) - \sigma(x - s) \). Differentiation with respect to \( x \) yields, for a fixed value of \( \sigma \), the relation \( \partial W(x)/\partial x = \sigma \). On the one hand, this relation allows us to calculate \( W(x) \) by direct integration of \( \sigma \); see equations (3.1) and (3.2). On the other hand, this relation allows us to find \( x \) for a given value of \( \sigma \) from the minimum of the function \( W(x) - \sigma(x - s) \). Under the condition where there is only one possible phase, this function has its minimum at \( x = s \). However, because there are two possible configurations for the molecules (α and WLC), this equation can have two solutions. This is illustrated in figure 6b. The total energy \( W(x) - \sigma(x - s) \) is the difference between the curve \( W(x) \) and its tangent as \( x = s \). For small (green curve) and large (red curve) values of \( s \), this is positive for all values of \( x \) and, thus, its minimum is at \( x = s \). For intermediate values of \( s \), however, a linear combination of α-phase and WLC, as given by the convex envelope of the energy curve (broken line), is smaller than the value \( W \) at \( x = s \). This means that the coexistence of molecules in α and in WLC configurations is more favourable than either phase on its own. This is completely analogous to the Gibbs double-tangent construction for the coexistence of two phases [35]. The slope of the double tangent corresponds to the constant stress \( \sigma_y \), where the amount of WLC (β*) gradually increases at the expense of the α-phase. Noting that the stress \( \sigma \) is just the slope of the \( W(x) \) curve, for all \( \sigma < \sigma_y \) the α-phase is stable (the green portion of the curve on the left side), and for all \( \sigma > \sigma_y \) the β*-phase is stable (the red portion on the right side). Hence, the transition occurs at \( \sigma = \sigma_y \).

The parameters of the model were chosen so that the mechanical behaviour exactly fits the SAXS observations for the WECs. In particular, we chose: \( s_0 = 100 \text{ nm}, \ K N / A_0 = 7.18 \text{ MPa nm}^{-1}, \ L = 259 \text{ nm}, \ C = (kT/l_p^\alpha)(N/A_0) = 3.93 \text{ MPa} \). These values end up fitting quite well with the proposed initial coiled-coil configuration. For example, the maximum observed extension for single molecule stretching of a myosin coiled-coil is 2.5 times higher than the initial length [12]. Additionally, we find that the calculated persistence length falls within a reasonable range. In order to convert the stress \( \sigma \) in figure 6b to the force on a single molecule, we use the lateral spacing determined by WAXD and assume a roughly hexagonal packing in cross section to estimate the area per molecule as \( A_0 / N \approx 0.1 \text{ nm}^2 \). At room temperature and taking the numerical values reported in the paper, we obtain a value for the persistence length \( l_p^\alpha \approx 9.8 \text{ nm} \). This is smaller than for DNA and an α-helix, but somewhat larger than a completely flexible protein [12,36] possibly owing to the tight packing of the chains. It is also probably an overestimate because every molecule not pointing in the stress direction will reduce \( N \) and, thus, reduce \( l_p^\alpha \). With these numerical values, it appears clearly in figure 6c that in the low strain phase, all the molecules are initially in the α-phase (green line). At a critical stress of \( \sigma_y = 6.7 \text{ MPa} \), we observe a coexistence of α- and β*-phase. This remains true until the strain has reached a value around 40 per cent at which point all the molecules are in the β* state. Further extension is easier than in the α-phase (as clearly shown by the reduced slope of the red line in figure 6c, when compared with the green line) because of the comparatively soft behaviour of WLCs at these strain values. Moreover, WLCs have a nonlinear elastic behaviour as visible in the figure, which is consistent with experimental strain behaviour observed in SAXS. Of course, this is just an equilibrium model that neglects any time-dependent effects, and a more detailed model must be developed in order to describe the hysteretic behaviour during relaxation. Nevertheless, it is in good agreement with the SAXS data during stretching of the WEC biopolymer.

4. CONCLUSIONS

Similar to man-made packaging materials, a primary function of the WEC appears to be prolonging the ‘shelf-life’ of the embryos until they can hatch. In this study, we combined synchrotron-based WAXD
and SAXS, as well as polarized confocal Raman spectroscopy with in situ tensile testing to correlate changes in protein secondary structure and supramolecular structure of the WEC to mechanical properties in real time. By correlating the structural information extracted from each technique with the in situ mechanical behaviour (figure 5), several important concepts emerge. Briefly stated, they are the following. (i) Stretching of the WEC tissue results in the discontinuous transition between distinct structural phases during yield that we identify as α-helical and βa, respectively. The coexistence of the two phases during yield suggests a classical phase transition rather than a gradual extension of the backbone. (ii) Unlike keratin and hagfish slime, the initial α-helical coiled-coil phase in the WEC is not transformed into a β-sheet phase (up to true strain values of 70%). This likely contributes to the stark differences observed in the mechanical behaviour of the different tissues. (iii) The transition between the α- and βa-phases during yield is completely reversible; however, there is a conformational hysteresis associated with the relaxation at all three hierarchical levels examined. Hysteresis exists only within the yield region, where there is a coexistence of the α- and βa-phases. (iv) Pre-yield and post-yield (low-strain and high-strain) behaviour of the protein chains in the WEC tissue is elastic. In the pre-yield region, this results from the stretching of the hydrogen-bonded α-helical chains. In the post-yield region, this results from the entropic deformation behaviour in the peptide backbone of the WLCs in the βa-phase. The hysteretic behaviour and the important role of classical phase transitions in determining this behaviour bears a striking similarity to pseudoelastic materials [4]. While pseudoelasticity has been observed in numerous alloys at moderate strain values (less than 10%), as far as we know, it has never been identified in a polymer with high extensibility, prompting efforts for an even deeper understanding of the molecular-level mechanisms and underlying physics of WEC behaviour.

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