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Life in extreme environments: Single molecule force spectroscopy as a tool to explore proteins from extremophilic organisms

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ABSTRACT

Extremophiles are organisms which survive and thrive in extreme environments. The proteins from extremophilic single-celled organisms have received considerable attention as they are structurally stable and functionally active under extreme physical and chemical conditions. In this short article, we provide a short introduction to extremophiles, the structural adaptations of proteins from extremophilic organisms and the exploitation of these proteins in industrial applications. We provide a review of recent developments which have utilised single molecule force spectroscopy to mechanically manipulate proteins from extremophilic organisms and the information which has been gained about their stability, flexibility and underlying energy landscapes.

INTRODUCTION

Life on Earth has adapted to exist in a vast range of environments, including the ice caps of the polar regions, deserts and on the ocean floors [1]. While human life exists within a relatively narrow window of environmental conditions, there are organisms which survive and thrive without difficulty in environmental extremes [2]. Such organisms are known as extremophiles and it is now difficult to find a place on Earth bereft of life. Particularly well adapted to these extreme environments are single-celled organisms from the Archaea and Bacteria [3], which can be found thriving in boiling water, alkaline lakes, acid rivers and under very high pressures [4]. Some extremophiles can tolerate high levels of radioactivity as well as living without oxygen or sunlight, instead obtaining their energy from sulphur or hydrogen. These organisms have raised interesting questions about the natural limits of life, as well as fuelling discussions on the origin of the first cell and the possibility of life on other planets [5]. Furthermore, these organisms and their constituent biological components have enormous potential for applications in biotechnology, including bioremediation, healthcare and energy production [6].

The proteins from extremophilic organisms have played a key role in enabling them to survive and function in specific environmental extremes [7]. For example, the proteins from hyperthermophilic organisms exhibit unusually high heat resistance and contribute to the ability of the organism to live in environments at temperatures above 80 $^{\circ}$C [8]. Proteins from extremophilic organisms are therefore of great interest as they have the ability to retain their folded structure and to possess the necessary flexibility to complete their function, under

conditions which denature proteins that lack these adaptations. For this reason, they offer attractive model systems in which to explore the origin of protein structure and dynamics under different conditions.

This mini-review begins with an introduction to some of the extremophilic organisms found on Earth followed by a description of the ways in which proteins from these organisms are being exploited in industry. Next, the structural adaptations of proteins from extremophilic organisms compared to their mesophilic counterparts are considered. An introduction to the experimental technique of single molecule force spectroscopy as a tool to explore proteins from extremophilic organisms is then given, including a focus on recent work in which a protein from a hyperthermophilic organism has been studied. The mini-review ends with a short discussion on possible future directions for this area.

DISCOVERY OF LIFE IN EXTREME ENVIRONMENTS

The first recorded discovery of life in an extreme environment can be attributed to the American microbiologists Thomas and Louise Brock who isolated the organism Sulfolobus acidocaldarius from a hot, acidic, sulphur-rich geothermal pool in Yellowstone National Park in 1965 [9, 10]. Brock successfully cultured the bacterium at a temperature of 95 °C, the temperature of the pool from which it had been extracted [11]. Brock's discovery of an organism that grows at such high temperatures, the first extremophile, initiated the hunt for more microbes in other environmental extremes and began the still-growing list of discoveries [12]. Extremophiles have now been found in nearly every region of the planet, from active glaciers in Alaska, the alkaline-rich waters of Mono Lake in California, to the hot, hydrothermal vents of the Pacific Ocean and the acidic water of the Rio Tinto in Spain (Fig. 1). Each discovery has provided more insight into the diversity of organisms in extreme environments and offers opportunities to understand how these organisms are adapted for survival.

Extremophilic organisms are classified on the basis of the particular extreme environmental condition in which they live [1]. The Sulfolobus acidocaldarius organism, first discovered by Brock, is classified as a hyperthermophile as it has an optimum growth temperature above 80 °C [9, 10]. Other extreme temperature-related extremophiles include thermophiles (optimum growth between 60 and 80 °C) and psychrophiles (optimum growth below 15 °C) [8, 13]. Non-extremophiles, which are termed mesophilic organisms or mesophiles, have an optimum growth temperature between 15 and 60 °C.

As well as extremes of temperature, some Archaea and Bacteria live in very acidic or alkaline environments [1]. Acidophiles exist below pH 5 and have been found in the hot springs of geothermal vents, where sulphurous gases dissolve in water to produce sulphuric acid, as well as in the acid waters that leach from metal and coal mines [14]. Conversely, alkaliphiles favour conditions above pH 9, such as Mono Lake in California which has a pH of 10 [15].

Halophiles are organisms that live in environments with high salt concentrations and are classified by the extent of their salt requirement for growth, including slight halophiles (0.3 – 0.8 M NaCl), moderate halophiles (0.8 – 3.4 M NaCl), and extreme halophiles (3.4 – 5.1 M NaCl) [16]. Halophiles require salt for growth, while halotolerant organisms do not require salt but can grow under saline conditions. Non-halophilic organisms grow optimally in conditions with concentrations of 0.2 M NaCl and below [16].Algae, bacteria and archaea have been found flourishing in the high salt concentrations of the Dead Sea, which has a salt content of 28 %

w/v (equivalent to 4.8 M NaCl). Most of these organisms are obligate halophiles and cannot tolerate salt concentrations of less than 15 % w/v (equivalent to 2.6 NaCl). Piezophiles, which are also known as barophiles, are organisms which display optimum growth pressures above 40MPa such as at the depths of the ocean floors[17].

EXPLOITING THE POTENTIAL OF PROTEINS FROM EXTREMOPHILES

Proteins extracted from extremophiles can tolerate extremes of temperature, salt, acid, and pressure, all of which have relevance in biotechnological research and industry[6]. The first enzyme to be extracted from an extremophile and commercially exploited was the hyperthermophilic enzyme Taq polymerase which was isolated from the archaea Thermophilus aquaticus [18]. This organism was another of Brock's early discoveries of extremophiles, also found in the superheated geothermal pools of Yellowstone National Park. Taq polymerase is used to make multiple copies of DNA, and is now routinely employed in the polymerase chain reaction (PCR) process which has become an indispensable workhorse of modern molecular biology [19].

Extremophilic organisms offer a rich source of naturally tailored, robust enzymes that are able to withstand harsh conditions in industrial processes that were long thought to be destructive to proteins [6]. Enzymes extracted from psychrophiles are used for effective soap and detergent use at low temperatures[20]. They are also used as antimicrobial targets in food preservation, in the agricultural industry for modifying crops to protect them from cold weather conditions, and to achieve efficient rates of enzymatic reactions at lower temperatures to improve costs in the baking industry. Acidophiles are used in the recovery of valuable metals such as gold and copper from low grade ores, in a process known as microbial leaching [14]. More generally, Archaea and Bacteria are increasingly exploited in bioremediation projects and to degrade toxic compounds in industry. While there are many examples of the use of proteins from extremophiles, we are only beginning to explore their exploitation and the potential applications are vast [6]. The advancement of this research relies on a detailed understanding of the structural adaptations of proteins from extremophiles and knowledge of the resulting stability, function and dynamics of these molecules under different environmental conditions.

STRUCTURAL ADAPTATIONS IN PROTEINS FROM EXTREMOPHILES

Much valuable work has been done to understand the structural adaptations of proteins from extremophiles [7]. By comparing high-resolution structural data of homologous proteins from mesophilic and extremophilic organisms, a large degree of secondary structural similarity has been found. Interestingly, subtle differences in the sequences of proteins often result in huge variability in thermostability [21]. Structural observations reveal a progressive pattern of stabilisation of proteins from extremophilic organisms through multiple additional interactions at solvent exposed, loop and interfacial regions [22]. For example, comparative studies of the structure and thermodynamic stability of proteins of mesophilic, thermophilic and hyperthermophilic organisms have revealed the importance of ionic interactions and

networks, increased hydrophobicity, an enhanced packing density and increased numbers of well-distributed electrostatic interactions[23].

While there is now a wealth of information on the structural adaptation of proteins from extremophilic organisms, there is comparatively less quantitative information on their conformational dynamics and flexibility. Detailed examination of the interplay between conformational dynamics and the solvent environment is difficult using conventional techniques that average the properties of many molecules. In the present review we provide an introduction to an approach which provides information about the stability and unfolding and folding kinetics of single protein molecules. In this mini-review we can only focus on one approach, but it should be noted that there are others which include NMR studies on conformational proteins dynamics [24] and FRET experiments [25]. By examining single molecules one at a time, the individual dynamics of subpopulations can be measured. One such way to examine proteins is to perturb single protein molecules using force.

USING FORCE SPECTROSCOPY AS A TOOL TO EXPLORE SINGLE PROTEINS

Single molecule force spectroscopy using an atomic force microscope (AFM) is a powerful tool to mechanically manipulate single proteins [26-28]. Using an AFM, a force can be applied to extend and unfold a single protein at a constant velocity, yielding information on the mechanical stability of the protein, or the force required to unfold it (Fig. 2). In the last two decades this approach has been used to measure the mechanical stability of a large number of natural and designed proteins, providing insight into the relationship between protein structure and stability as well as directly measuring protein unfolding and folding kinetics [26]. Such AFM studies have shown that the mechanical stability or robustness of proteins can be ranked according to their secondary structure content and arrangement; α-helical proteins generally exhibit lower mechanical stability than proteins with a high β-sheet content, and in β–strands the shearing apart of hydrogen bonds requires more force than the sequential un-zipping of hydrogen bonds [29-31]. The effects of side chain packing and long-range interactions in topologically similar proteins [32], hydrophobic packing in the hydrophobic core of a protein [33], solvent accessibility of hydrogen bonds in the protein, and specific hydrogen bond network motifs [34, 35] have been examined using this approach. This insight into the role of specific intra- and inter-molecular interactions at the single molecule level provides an unrivalled opportunity to resolve the molecular determinants of protein stability. Applying such a technique to the study of proteins from extremophiles will allow for a direct test of the relationship between structural adaptations, and the resulting intra- and inter-molecular interactions, protein stability and dynamics.

SINGLE MOLECULE STUDIES ON A PROTEIN FROM A HYPERTHERMOPHILIC ORGANISM

The ability of proteins from hyperthermophilic organisms to maintain their native structure, yet be dynamic and flexible is a key determinant of their ability to function at the extremes of environmental temperatures found on Earth [8]. Variable-temperature single molecule AFM has been employed to mechanically manipulate a protein from a hyperthermophilic organism, as a function of temperature [36]. The cold-shock protein (TmCsp) from the hyperthermophilic bacterium, Thermotoga maritima, has a β-barrel structure and has been well characterised structurally and thermodynamically [21, 37-40]. The cold-shock protein belongs to a sub-set of

the OB (oligonucleotide / oligosaccharide-binding) class of folds, a protein fold that is found in all three kingdoms of life, and is often but no always up regulated by a sudden decrease in temperature as part of a cold shock response.

Protein engineering was used to generate constructs that incorporate the TmCsp protein domain within a chain of mechanically well-characterised marker protein domains [37]. These constructs are known as chimeric polyproteins [26]. The marker proteins in the chimeric polyprotein provide a clear mechanical fingerprint that a single polyprotein construct has been picked up by the AFM tip, and inspection of force-extension experimental traces allows for the identification of mechanical fingerprints for TmCsp (Fig. 3A). Experiments on the chimeric polyprotein, $(I27\text{-}TmCsp)_3$-I27, revealed two populations of mechanical unfolding events (Fig. 3A and B). One population corresponds to the previously well-characterised I27 protein, while the other reveals the mechanical fingerprint for TmCsp [37].

Using this approach, the mechanical stability of TmCsp was characterized in the temperature range of 5 – 40 ˚C [36]. While the mechanical signature of TmCsp is maintained over the entire temperature range, the force required to unfold the protein decreases with increasing temperature (Fig. 3C). Temperature-dependent changes in features of the unfolding energy landscape of this protein have been measured by studying the pulling speed dependence of the unfolding force with temperature in combination with Monte Carlo simulations. The change in free energy barrier to unfolding is small and within experimental uncertainty, and the main effect of temperature is seen in the distance between the native, folded state of the protein and the unfolding transition state, Δx_U (Fig. 3D). The Δx_U of TmCsp is already large and increases with increased temperature, reflecting a reduction in the spring constant of the protein and an increase in the malleability or deformability of the structure. Malleability within the native folded basin is contrary to the hypothesis that proteins from hyperthermophiles should have rigid structures resulting from improved packing and increased numbers of ionic interactions [8]. Instead, these experiments suggest that enhanced malleability/deformability at higher temperatures may enable proteins from hyperthermophilic organisms to maintain and easily reform their structure when exposed to denaturing high temperature conditions [36].

FUTURE OUTLOOK

The mechanical robustness and malleability of TmCsp provides an insight into the stability and dynamical properties of hyperthermophilic proteins. This work lays the foundation for further studies of extremophilic proteins. For example, it will be interesting to uncover details of the folding energy landscape of proteins from extremophilic organisms and to understand the importance of folding intermediates and the ruggedness of the energy landscape (Fig. 3E). By employing a single molecule approach, detailed insight can be gained into the connection between protein stability and flexibility and features of the energy landscape can be accessed.

Given the existing applications of proteins from extremophiles it is interesting to consider how the newly measured mechanical properties of TmCsp could be exploited. Mechanical properties are among the most fundamental requirements of biomaterials. Proteins are currently being used as building blocks in the design of robust biomaterials, as they possess the desired elasticity, mechanical robustness and flexibility required for functional materials [41-43]. The use of proteins in the rational design of biomaterials requires a detailed understanding of their nano-scale and micro-scale mechanical properties, now readily accessible using AFM force spectroscopy. In fact, the mechanical stability and

malleability of a wide range of proteins has now been measured using this approach (Fig. 3F). To fully exploit proteins as self-assembling components in the design of new materials it will be necessary to expand the tool-box of proteins available. While a wide range of biomaterials have been developed using natural proteins and synthetic polymers, it remains challenging to design advanced materials that are both thermodynamically and mechanically robust and possess the malleability or rigidity to be useful for function. Proteins from extremophilic organisms therefore present interesting opportunities to rationally engineer or re-engineer robust biological materials for exploitation.

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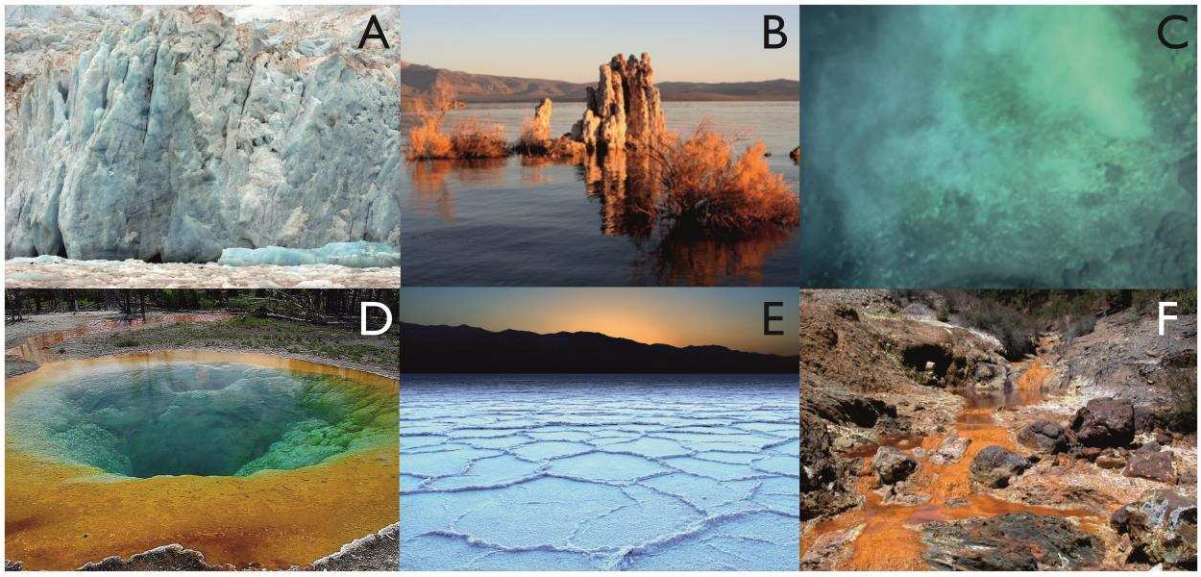

Figure 1: Examples of extreme environments (A) Chenega glacier, an active glacier in Alaska (image credit: U.S. Fish and Wildlife Service); (B) A calcium carbonate spire formed by the interaction of fresh spring water and the alkaline water of Mono Lake, California (image credit: Mila Zinkova); (C) Hot hydrothermal fluids and gases venting from the sea floor, Western Pacific Ocean (image credit: National Oceanic and Atmospheric Administration, U.S.A.); (D) Morning glory, acidic hot spring in Yellowstone National Park, U.S.A. (image credit: Jon Sullivan); (E) Badwater salt flats, California (image credit: Dave Toussaint); (F) The acidic Rio Tinto river in Spain (image credit: Carol Stoker, The National Aeronautics and Space Administration, U.S.A.).

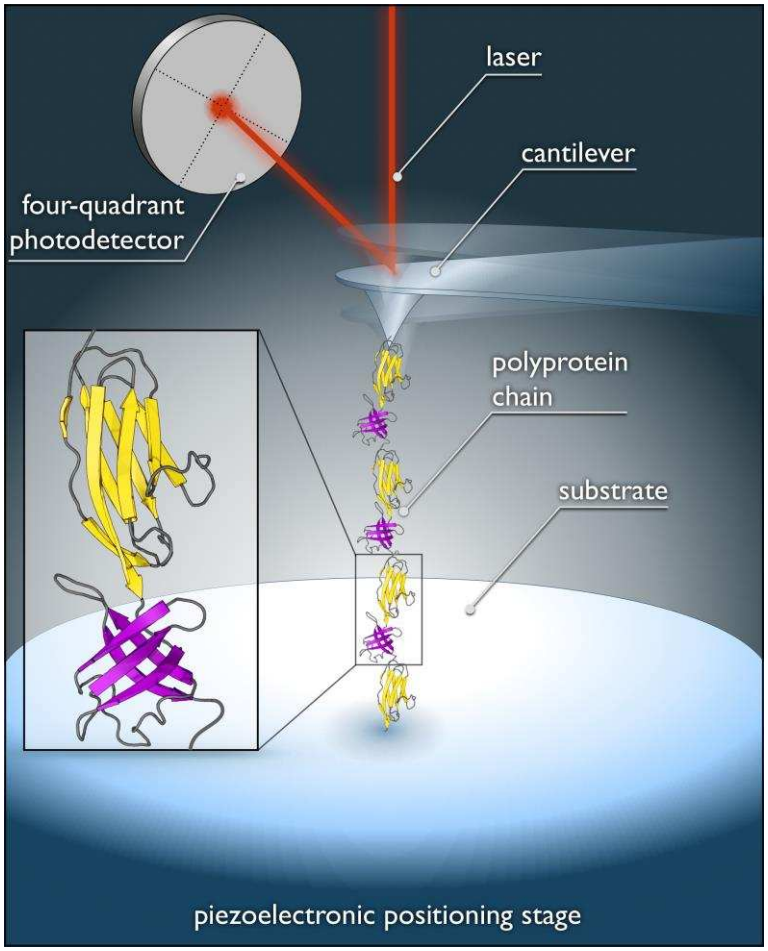

Figure 2 Using the atomic force microscope (AFM), a single polyprotein is stretched between the tip of a flexible cantilever and a flat substrate mounted on a piezoelectric stage. When the tip and substrate are brought together, a polyprotein can attach to the tip by adsorption. The substrate is then withdrawn at a constant velocity, increasing the distance between the tip and substrate and resulting in the extension of the molecule. This generates a restoring force that causes the cantilever to bend. The bending of the cantilever is measured using a laser beam which is directed towards the upper surface of the cantilever. The deflection of the cantilever can then be detected using a photodetector. The output of the photodetector can be related to the movement of the cantilever and therefore to the applied force, if the elastic properties of the cantilever are known. This system allows spatial manipulation of less than a nanometer and can measure forces of only a few pico Newtons up to hundreds of pico Newtons.

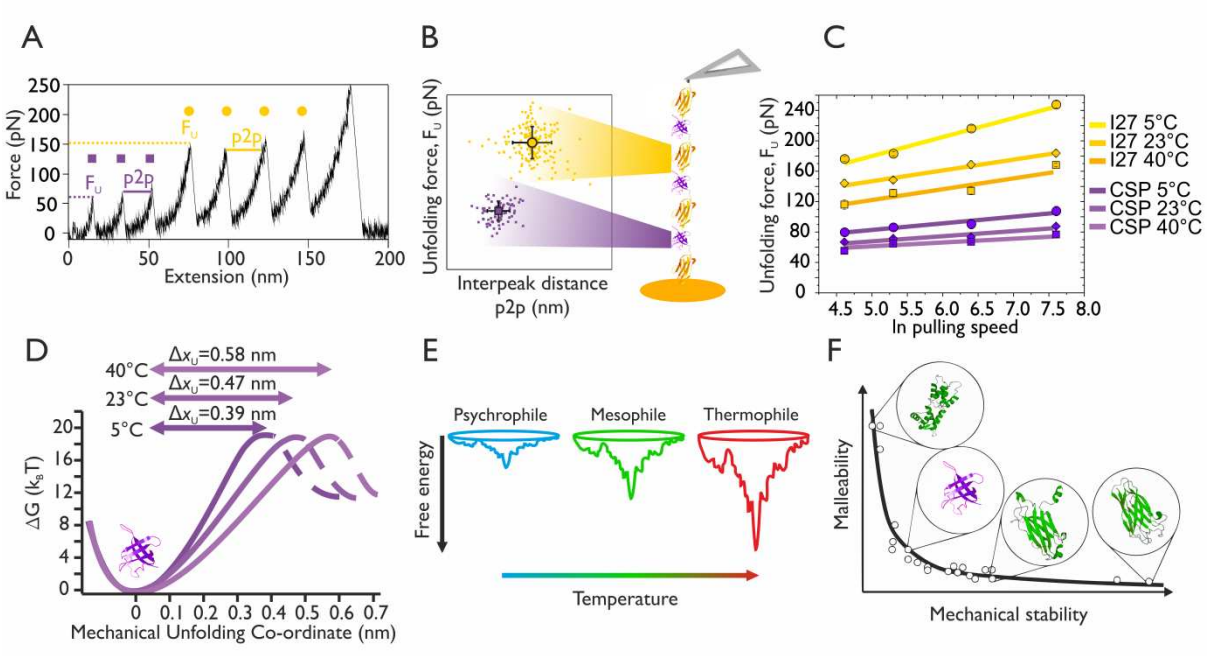

Figure 3 A) An example AFM experimental force-extension trace showing the unfolding of a full polyprotein chain containing three TmCspdomains (purple squares) and four I27 domains (yellow circles) at room temperature, and a pulling speed of 600nms^{-1}. The measured peak unfolding forces (F_U) and inter-peak distances (p2p) for each unfolding event are recorded. B) Scatter plot of inter-peak distances and peak unfolding forces for TmCsp and I27 from a single experiment at 600nms^{-1} at room temperature. The median F_U and p2p values are plotted as symbols with black outlines. C) Each experiment is repeated in triplicate, at four different pulling speeds. The average of the three median F_U values for I27 and TmCsp for each experiment are each shown as an individual symbol. There is a linear dependence of F_U on the ln of the pulling speed over this range of pulling speeds. This enables the data to be fitted using the Bell-Evans-Ritchie model to obtain basic parameters of the mechanical unfolding energy landscape of each protein. D) The two-dimensional mechanical unfolding energy landscape of TmCsp at three different temperatures, illustrating the "temperature softening" effect, where the distance from the native, folded state of the protein and the energy barrier to unfolding increases with increasing temperature reflecting a more malleable structure. E) Schematic depicting the energy funnel model of protein folding for proteins from psychrophilic, mesophilic and thermophilic organisms. The lowest free energy state corresponds to the most stable, native, folded state of the protein. The higher energy states correspond to random-coil and unfolded protein structures. Proteins from psychrophilic organisms are generally less stable than their mesophilic and thermophilic counterparts, meaning that it is easy for them to interchange between different structures. This is an essential adaptation to enable them to function at lower temperatures. The ruggedness of the bottom of the energy funnel depicts the energy barriers for inter-conversion or structural fluctuations of the native state. F) The relationship between the malleability and the mechanical stability of proteins with different secondary structure content. Typically, a protein with a greater percentage of alpha-helical secondary structure is more malleable but less mechanically stable than a protein with a greater percentage of beta-sheet structure. Figures 3A & B are reprinted with permission from Journal of Physical Chemistry B, 117(6): 1819-26 (2013). Copyright 2013 American Chemical Society. Figures 3C & D are reproduced from Ref. Soft Matter, 9(37), 9016-9025 (2013) with permission from The Royal

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