Nanostructured functional films from engineered repeat proteins

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Fundamental advances in biotechnology, medicine, environment, electronics and energy require methods for precise control of spatial organization at the nanoscale. Assemblies that rely on highly specific biomolecular interactions are an attractive approach to form materials that display novel and useful properties. Here, we report on assembly of films from the designed, rod-shaped, superhelical, consensus tetratricopeptide repeat protein (CTPR). We have designed three peptide-binding sites into the 18 repeat CTPR to allow for further specific and non-covalent functionalization of films through binding of fluorescein labelled peptides. The fluorescence signal from the peptide ligand bound to the protein in the solid film is anisotropic, demonstrating that CTPR films can impose order on otherwise isotropic moieties. Circular dichroism measurements show that the individual protein molecules retain their secondary structure in the film, and X-ray scattering, birefringence and atomic force microscopy experiments confirm macroscopic alignment of CTPR molecules within the film. This work opens the door to the generation of innovative biomaterials with tailored structure and function.

1. Introduction

Self-assembly that relies on highly specific biomolecular interactions is an attractive approach for the bottom-up design of biomaterials with sophisticated properties. Exploiting the same set of tools and interactions that exist in biology garnered interest as novel routes to materials with numerous potential applications in synthetic biology and nanobiotechnology [1–3]. In spite of our increased understanding of interactions that govern assemblies in nature, in the laboratory, there is still a challenge to make controlled assemblies that span scales over several orders of magnitude. Self-assembling materials based on peptides, proteins and nucleic acids have recently been explored [2,4–14]. In this work, we focus on protein materials because proteins provide a large repertoire of interactions and chemical reactivities that can in turn provide function to the materials [15]. We use protein design as a powerful tool to tune structure, stability and function of proteins for specific applications. Current technologies are such that we can readily produce large quantities of homogeneous and monodisperse protein material using bacterial recombinant expression systems [16,17]. Here, we present designs for self-assembling functional protein arrays.

Repeat proteins are an especially attractive target for nanotechnology applications owing to their hierarchical and modular structures. Our repeat module of choice is the 34 amino acid helix-turn-helix tetratricopeptide repeat (TPR) [18]. Naturally, TPRs occur in tandem repeat arrays, from three to 20, and their cellular role is assembly of multi-protein complexes [18,19]. Here, we use a protein based on an idealized TPR consensus sequence (CTPR). Consensus sequence design has emerged as a protein design tool to create de novo proteins that capture sequence–structure relationships and interactions present in nature. CTPR repeats can be combined in tandem to form superhelical arrays, in which eight repeats comprise one full turn of the superhelix (figure 1a)
We tested this proposition by recording circular dichroism (CD) wavelength spectra of the films. As figure 2a indicates, spectra characteristic of alpha-helical secondary structure are observed for CTPR18 films, with no evidence that there is any significant structure other than alpha-helix. Even though the CD spectrum of the solid film cannot provide quantitative information (the protein concentration cannot be estimated), any significant change of structure would be evident in the spectrum. Moreover, the spectrum of the CTPR18 in the film is practically indistinguishable from the spectrum of the protein in solution (figure 2a, inset). These observations confirm that CTPR18 protein maintains its secondary helical structure in the film and does not undergo unfolding during the assembly. In addition, self-assembly of CTPR18 into films does not involve amyloid-like fibre formation as was previously observed for protein self-assembling films [5]. In this case, the solvent casting process does not disrupt the highly regular atomic-level structure, and most probably the packing of CTPR18 superhelices is the driving force for self-assembly.

We then generated a CTPR film in the presence of 1:10 peptide DESVD:protein molar ratio to ensure the absence of free peptide ligand in the film. The N-terminus of the peptide is labelled with fluorescein, which allows us to monitor the anisotropy of the peptide–ligand in the film relative to the free peptide in solution. If CTPR helices are macroscopically aligned, peptide-binding sites along CTPR will also be aligned in the film. Thus, we expect strong anisotropic signal from the peptide bound to the film that depends on the orientation of the film in respect to excitation and emission slits. Figure 2b shows the change in fluorescence intensity of the peptide ligand when the emission polarizer is rotated from 0° to 360°. The signal shows clear maximum and minimum values, which indicates the anisotropy of the sample. The data can be well fitted to a sine wave function with maximum to minimum peak distance of 100°. This result shows that the proteins retain their biological activity, i.e. ligand binding, within the solid material and that molecular recognition in such materials can be used to impose ordering to otherwise isotropic fluorescent peptides.

Further structural characterization of CTPR films using polarized optical microscopy indicated that CTPR18 films

[20,22]. The stability and ligand-binding properties of CTPRs can be modulated in a predictable manner [23–25].

In the crystal structures of CTPR arrays, individual molecules stack head-to-tail to form virtually continuous superhelices, in which the inter-molecular packing interface is identical to the intra-molecular repeat–repeat interface (figure 1a) [20,21]. Therefore, we hypothesized that under certain conditions CTPR proteins may self-assemble into ordered films. Figure 1a,b schematically illustrate a hypothetical macroscopic alignment of CTPR superhelices. According to the schematics, higher order structures may assemble by specific contacts between superhelices, head-to-head and side-to-side, which may be similar to the ones present in the crystalline forms. In addition, if individual protein molecules retain their secondary structure in the film, proteins will also retain their specific ligand-binding function.

The CTPR18 protein used in this study contains three designed binding sites for peptide sequence DESVD [26]. Three tandem CTPR repeats (CTPR3) display one peptide-binding site on its surface [figure 1c], the affinity and specificity of which can be engineered at will [26–29]. CTPR18 protein is made out of six CTPR3 modules, where three peptide-binding modules alternate with three so-called spacer modules [figure 1c]. CTPR18 was expressed and purified as previously described [30]. Protein solutions, 3% w/v CTPR18 in 10 mM NaCl, 10 mM Na phosphate pH 7.0 buffer and 1% w/v poly(ethylene glycol) (PEG) 400, were deposited on Teflon tape. After solvent evaporation at room temperature, solid ‘macroscopic’ films are formed. The multi-layered films, of the order of 100 µm thick, were removed from the Teflon surface for further characterization. It has been shown previously that addition of small organic molecule ‘plasticizers’, such as PEG, to the liquid crystal-like assemblies of biomolecules causes additional ordering [5,31]. We observed that CTPR films can be assembled without addition of plasticizer, but those films are more brittle than ones assembled in the presence of 1 per cent PEG 400.

If the solid films are a consequence of the self-assembly of CTPR superhelices, we would expect that the proteins would retain their characteristic alpha-helical secondary structure. We tested this proposition by recording circular dichroism (CD) wavelength spectra of the films. As figure 2a indicates, spectra characteristic of alpha-helical secondary structure are observed for CTPR18 films, with no evidence that there is any significant structure other than alpha-helix. Even though the CD spectrum of the solid film cannot provide quantitative information (the protein concentration cannot be estimated), any significant change of structure would be evident in the spectrum. Moreover, the spectrum of the CTPR18 in the film is practically indistinguishable from the spectrum of the protein in solution (figure 2a, inset). These observations confirm that CTPR18 protein maintains its secondary helical structure in the film and does not undergo unfolding during the assembly. In addition, self-assembly of CTPR18 into films does not involve amyloid-like fibre formation as was previously observed for protein self-assembling films [5]. In this case, the solvent casting process does not disrupt the highly regular atomic-level structure, and most probably the packing of CTPR18 superhelices is the driving force for self-assembly.

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are evenly birefringent (figure 2c). This observation suggests that macroscopic alignment of CTPR18 is achieved and is uniform over the entire film. Macroscopic ordering was further observed in the X-ray diffraction pattern of the CTPR18 films. For macroscopically aligned materials, the diffraction pattern has discrete features that are dependent on the angle of the incident X-ray beam. We acquired diffraction patterns of the protein films at room temperature while rotating the sample with a constant sample–detector distance.

Figure 3 shows the diffraction patterns at 0°, 90° and 180° which present three main noticeable features: (i) diffraction arcs, characteristic of macroscopic alignment; (ii) dependence of the diffraction arcs on the angle, characteristic of a directional order; and (iii) presence of the diffraction pattern mirror image when the sample is rotated by 180°, as expected for all the elements being aligned along a common axis. These results provide clear evidence of the order present in the solid films. The two main diffraction arcs observed on the X-ray diffraction pattern at 0.43 nm can be attributed to the helical pitch of the alpha-helices, providing an additional confirmation of the helical structure observed by CD. Moreover, the phase determined by the X-ray diffraction experiments of approximately 90° is in agreement with the phase obtained from the fluorescence anisotropy experiments, indicating that macroscopic ordering pattern of the film is indeed imposed on the ligand that is otherwise isotropic.

We additionally used atomic force microscopy (AFM) to characterize the topography and roughness of the protein films. We imaged both the film surface and the film fracture. As is evident from the AFM image of the film in figure 4a, the surface of the film is very smooth. Because of the tight packing between the CTPR arrays, individual protein molecules or their packing arrangement in the film cannot be observed by AFM. The AFM image indicates a flat, smooth surface with a very small roughness of about 1.25 nm, comparable with previously described roughness of uniform films formed of proteins [32] or other biomolecules [33]. In the images of the film freeze-fracture elongated, repetitive structures are present that are not observed on the surface of the film (figure 4b) additionally confirming anisotropic structure of the films consistent with superhelices being aligned along a common axis.

The details of the experiments described are given in the electronic supplementary material.
consensus TPRs. We generate solid films composed purely of TPR proteins in which the individual elements spontaneously self-assemble to generate ordered novel biomaterials. The repeat proteins in the solid state not only preserve their characteristic structure, but also maintain their functionality.

Previously, protein films have been generated from β-strand amyloid fibres of proteins [5,34]. In addition, coiled-coil forming peptides were also successfully rationally engineered to generate nanostructured fibrous materials [6]. Our work is the first example, to our knowledge, of a solid-state protein film in which not only the protein’s original structure but also its functionality are maintained. Thus, the information from the X-ray structure of CTPR arrays provides knowledge of the exact position of every atom in the array and the ability to predict their position within the higher order assemblies.

This work presents a novel route for generation of biomaterials with tailored structural and physical properties. Moreover, we demonstrated the ability of CTPR films to induce order to small molecules and peptide–ligands. Our experiments demonstrate that it is possible to introduce tailored functionalities at precise positions within the protein that will further propagate to the macroscopic dimensions through the protein’s regular self-assembly.

In the future, the introduction of novel reactive functionalities into the CTPR protein through the use of non-canonical amino acids will allow us to use powerful chemistries such as click chemistry and photochemistry to broaden the range of potential applications of the TPR films.

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2. Conclusions

Here, we present a bottom-up strategy to generate functional, ordered biomaterials using the intrinsic self-assembly properties of repeat protein molecules and specific non-covalent protein–peptide interactions. We explore the features of a well-characterized repeat protein system, the designed film fracture sample preparation. This research is financially supported in part by the European Commission International Reintegration grant (IRG-246688) and AMAROUT-COFUND Europe Programme.

References


