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Engineered and designed peptide-based fibrous biomaterials

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Abstract

Our increasing understanding of peptide and protein folding and assembly raises new possibilities for engineering novel self-assembling supramolecular structures and bio-inspired materials. Here we focus on peptides designed de novo, and natural systems that have been engineered to form extended protein fibres. Potential applications of such assemblies include the preparation of functionalised biomaterials for the development of new diagnostic devices, scaffolds to recruit cells for cell/tissue engineering, and templates for the assembly of inorganic materials.

Keywords: α-Helix; Amyloid fibril; β-sheet; Coiled coil; Fibre; Nanotechnology; Peptide; Protein; Protein design

1. Introduction

1.1. Natural fibrous-protein structures

Fibrous assemblies of peptides and proteins are found throughout biology where they perform a variety of functions [1,2]. For example, the main components of the internal cytoskeleton of eukaryotic cells—namely, intermediate filaments, actin filaments and microtubules—give shape and particular mechanical and viscoelastic properties to cells; provide structures against which force is generated for cellular locomotion and the active transport of cargo through the cell; and participate in assemblies that assist in the movement and separation of chromosomal DNA during cell division. Outside the cell, fibrous-protein components of the extracellular matrix—notably, collagens and elastic fibres—combine with carbohydrates and cell-surface cell-adhesion molecules to provide the glue that holds cells together is tissue, whilst allowing access of nutrients, movement of cells and the removal of waste.

1.2. The engineering and design of new peptide-based biomaterials

This wealth of natural examples provides bio-inspiration for engineering and designing novel fibrous biomaterials for potential applications in materials science and nanobiotechnology. Broadly speaking, work in this area can be split into two types: (1) the 'redesign and engineering' of natural molecules to produce new structural and functional materials, and (2) the 'rational de novo design' of new peptide and protein assemblies, and hence novel materials, from our knowledge of how protein structure relates to sequence.

Natural fibrous structures are usually of the order of nm thick and μm long. However, they are built from building blocks orders of magnitude smaller than this: usually with Å to nm dimensions and masses in the range 10–100,000 Da. However, most of the natural protein building blocks that make up biological fibrous assemblies are difficult to prepare, characterise and manipulate. Recent observations and novel designs for small self-assembling peptides that form extended fibrous structures have led to solutions to these problems and, hence, to considerable activity in this area. This emerging and rapidly growing area of peptide-based self-assembling fibres is the subject of our review. Specifically, we focus on the period from January 2000 to July 2003.

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1.3. Hydrogen-bonded peptide structures as building blocks for biomaterials

In addition to distinguishing redesign and de novo design of peptide assemblies and materials, we differentiate between fibres based on the two main types of hydrogen-bonded structure in peptide and protein structures, namely, the α-helix and the β-sheet, Fig. 1, [3]. This distinction is important and useful for the following reasons.

Peptides and proteins are linear polymers of α-amino acids, generally referred to as 'polypeptides'. The polymer chain itself is called the backbone and the chemistries associated with each amino acid that hang off the backbone are known as the side chains. The conformations of polypeptide backbones are restricted by steric clashes between backbone and side-chain atoms. These restrictions are summarised in the famous Ramachandran plot, which describes all possible polypeptide conformations in terms of two backbone dihedral angles, psi and phi. The repetition of similar combinations of psi and phi leads to local structures known as secondary structure elements. Just two of these—α-helices and β-strands—predominate in protein structures. In folded proteins the hydrogen-bonding potential of the polypeptide backbone must also be accommodated. In the α-helix this is done efficiently with the majority of the backbone NH and CO groups being internally hydrogen bonded. In contrast, internal hydrogen bonding in β-strands is not possible and, so, two or more β-strands pair via hydrogen bonds to form extended structures called β-sheets. Such sheets can close to form so-called β-sandwiches and β-barrels, or form extended structures of the type seen in amyloid fibrils and amyloid-like assemblies [3].

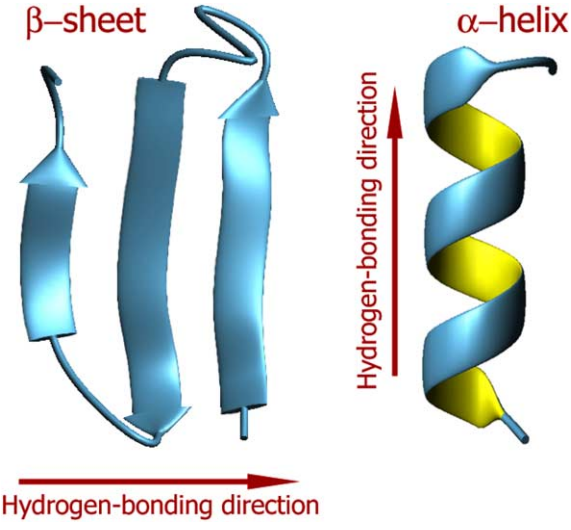

Fig. 1. Cartoons for the two main types of hydrogen-bonded structure in proteins, the α-helix and the β-sheet. Arrows show the hydrogen-bonding directions. In the α-helix, backbone hydrogen bonding occurs between backbone atoms of residues positioned i to $i+4$ along the polypeptide chain, making it a local form of (secondary) structure. β-Sheets, however, are made up of β-strands, which need not necessarily be close in the chain. A three-stranded anti-parallel β-sheet is shown.

With appropriate placement of hydrophobic (H) and polar (P) side chains along the polypeptide chain, both α-helices and β-sheets can be made amphipathic; that is, they can have distinct hydrophobic and polar faces. These features can be used to guide the self-assembly of α-helical and β-strand building blocks in prescribed ways. This is because hydrophobic faces cluster to avoid contact with water. Thus, amphipathic secondary structures assemble to bury their hydrophobic surfaces, whilst leaving their polar faces exposed to solvent. Because the two secondary structures have different periodicities their associated H–P patterns are different: HPHPHP-type patterns tend to guide the assembly of amphipathic β-strands and β-sheets, which may go onto form barrels and sandwiches; whereas HPPHPPP-type patterns lead to amphipathic α-helices that pack into bundles and rods.

For both structures, sequence-to-structure rules exist over and above the straightforward H–P patterns. Arguably, these are better developed for α-helical folding and assembly. Nonetheless, the field of peptide-based assemblies in materials science is currently more advanced for β-sheet-based and amyloid-like structures. This advance is due, at least in part, to a relatively recent and dramatic increase in knowledge about the assembly of fibrous structures based on β-sheets, which in turn reflects the immense medical interest in amyloid-like assemblies. For this reason we begin our review with recent studies of β-sheet-based fibrous structures.

2. β-Sheet-based and amyloid-like assemblies

Fibrillar materials assembled from proteins in their β-sheet form are widespread in nature. However, those most commonly recognised are amyloid and amyloid-like structures linked to a range of diseases of unrelated origin. These "amyloidoses" include Alzheimer's Disease [4], the transmissible spongiform encephalopathies [4], Parkinson's Disease [5] and type II (adult onset) diabetes [6], and may also encompass Huntington's Disease [7,8] and some forms of cataract [9]. Nonetheless and despite the lack of any underlying similarities in the sequences of proteins that form amyloid, there appears to be a common core structure adopted by the polypeptide chains within all of the fibrils associated with disease. This common structure is generally known as the "cross-β" structure and is being determined to increasing levels of resolution [10,11,*12,*13,*14]. In this configuration, individual polypeptide chains form β-strands that lie perpendicular to the long axis of the fibre, Fig. 2; the structures of two such strands in β-sheet

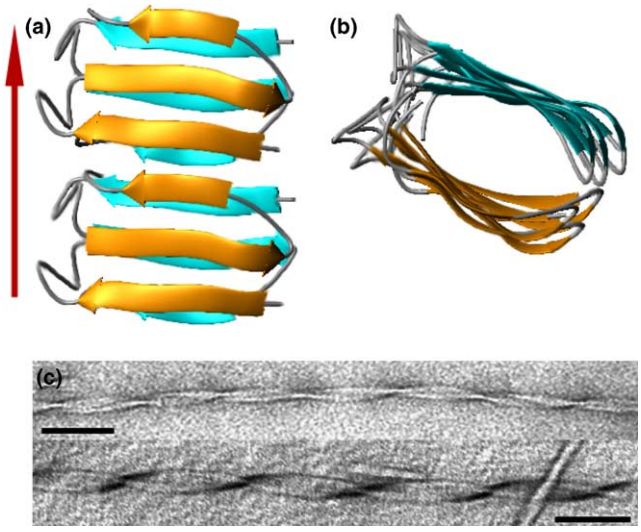

Fig. 2. (a,b) Orthogonal views of an idealised cartoon showing the fundamental structure of all β-sheet amyloid fibrils demonstrating the "cross-β" configuration. In (a) the arrow denotes the hydrogen-bonding direction. (c) Transmission electron micrograph of β-sheet fibrils assembled from insulin (*top*) and a mixture of two unrelated polypeptides (*bottom*). Note the regular helical twist repeat in both fibril species. The insulin fibrils are clearly assembled from filamentous (protofilament) substructures. The chirality of the twist cannot be determined from these images, however the twist is typically left handed. Scale bars: 100 nm.

fibrils have recently been determined [*13,*14]. Such strands are hydrogen bonded to form extended β-sheets that run parallel to the long axis. For instance, a recent model of the $A\beta_{1-40}$ peptide [*12,*14], which forms amyloid fibrils associated with Alzheimer's Disease, suggests that this particular polypeptide forms two β-strands linked by a bend to form a hairpin-like structure. These strands are not hydrogen bonded to each other, but rather to strands in adjacent molecules to form extended parallel β-sheets. In this way many polypeptides may oligomerise and contribute to the core structure. Similar structures have been proposed for both related and unrelated polypeptide systems, although short peptides are unlikely to form a hairpin and therefore probably only make up single strands in this extensively hydrogen-bonded core structure. Multiple copies of these so-called protofilaments bundle in helical arrays to form the mature fibrils. Though the precise number of the protofilaments varies, the dimensions of the fibrils tend to be similar and typically ~10 nm in diameter, Fig. 2. All of these features suggest that the cross-β structure is a generic structure accessible to all polypeptide chains regardless of their aminoacid composition or sequence [15–17,*18]. This possibility immediately suggests an almost limitless array of building blocks from which to assemble bio-inspired fibres with a wide variety of physico-mechanical properties for materials purposes. Moreover, the ability to assemble mixed fibrils, comprising more than one chemically distinct polypeptide chain [17,*18] should allow tuning of both structure and function.

2.1. Natural amyloid-like assemblies and inspiration for novel applications in materials science

Despite the possibilities for β-sheet-based fibrillar materials, a question remains as to whether they are likely to be of use as biological, or bio-inspired materials. However, evidence increasingly suggests that despite their association with pathological states, certain amyloid-like fibrils also have functional roles in nature. For example, the hydrophobins, a class of proteins found in filamentous fungi [19], and their analogues (the chaplins) found in filamentous bacteria [20,21], are involved in modulating the surface tension of the fluid environment inhabited by the organisms. One hydrophobin, SC3, decreases the normal surface tension of water, from 72 to 24 $mJ\,m^{-2}$ [22], which makes it one of the most surface-active proteins known. At the air–water interface, hydrophobins and chaplins self-assemble into amphipathic rod-like structures 4–10 nm in diameter with the physical and tinctorial characteristics of amyloid fibrils [23]. These structures self-associate laterally to form close-packed amphipathic membranes at air–water [24] or oil–water [25] interfaces, and on solid surfaces like Teflon [25]. On binding, hydrophobins change the physical properties of surfaces (e.g. Teflon becomes hydrophilic and filter paper hydrophobic), behaviour which might be exploitable in surface, cell and tissue engineering.

Another class of natural amyloid-like fibril is produced by *Escherichia* and *Salmonella* species [*26]. These 'curli' fibres are involved in the colonisation of inert surfaces and biofilm formation. A remarkable aspect of their production is the level of control imposed over fibril localisation and assembly: the products of six genes are required for correct fibril production at the cell surface with at least two of these being needed for chaperoning the prefibrillar protein to the site of assembly, thereby preventing inappropriate (and presumably toxic) fibril formation at locations en route. Harnessing or mimicking such mechanisms may enable the controlled deposition of fibrils at defined locations on solid surfaces for the directed assembly of new materials, three-dimensional architectures, or devices.

Further examples of naturally occurring functional amyloid-like fibrils are less convincing. Unlike the hydrophobins, chaplins, and curli fibrils, which are secreted into the external environment, these other proteins have required significant chemical or physical manipulation prior to analysis. Given that amyloid-like fibril formation often arises as a direct consequence of protein destabilisation [27], the use of harsh conditions to purify proteins from natural environments may inadvertently result in protein aggregation and the

formation of amyloid-like structures. With this caveat in mind, amyloid fibrils have also been implicated in the activity of the antifreeze protein from winter flounder [28]; suggested as components that enable fertilised embryos of annual killifish *A. limnaeus* to survive water stress [29]; and proposed as structural components protecting silkmoth oocytes [30].

Thus, there is increasing evidence that β-sheet amyloid-like fibrils play structural and functional roles in nature, suggesting their possible use as biomaterials with diverse functions. There is also evidence that β-sheet fibrils self-associate in a well-defined fashion to form higher-order assemblies, which may also be of significant interest for their physico-mechanical properties. The native silkmoth oocyte chorion, for example, is a lamellar structure thought to be analogous to cholesteric liquid crystal [30], and Aggeli et al. have observed liquid crystal phase behaviour in artificial systems in vitro [31]. Interestingly, some fibrils associated with disease states are packaged in vivo [32] into structures that share the optical properties of the semi-crystalline spherulites formed by polyethylene, and similar structures are formed in vitro by immunoglobulin light chains [33] and insulin (CEM, unpublished results).

2.2. Designed amyloid-like assemblies and their potential applications

In addition to high-order structures, many in vitro β-sheet fibrillar systems form aqueous gels. This raises possibilities for their use as hydrogels, which are integral to the cosmetics and other industries and biotechnologies such as drug delivery and release [34], microfluidics [35], and tissue engineering [36,37]. For example, Zhang and co-workers demonstrate the utility of self-assembling β-sheet fibrillar scaffolds in promoting cell adhesion and growth for tissue engineering [38]. The scaffold designs are based on short peptides with alternating hydrophobic and polar amino acids (the HPHPHP-type repeat motif described above). In the presence of salt, these form membranes that comprise fibrils with the structural and tinctorial properties of amyloid [39,40]. The group report a number of chemically different scaffolds synthesized using this design strategy (reviewed more extensively elsewhere [41]), which support the adhesion of mammalian cell lines [42]; promote neuronal cell adhesion, neurite outgrowth and active synapse formation [38]; and support chondrocyte growth and promote the synthesis of the extracellular matrix and, so, opens possibilities for cartilage repair [*43].

Interesting developments in this area from other groups include the design of a longer polypeptide, polyEAK9, that forms extremely stable β-sheet fibrils, which gel in response to changes in conditions [*44]. These so-called responsive gels and switchable systems carry additional benefits in drug-delivery and tissue-engineering applications and activity in this area is increasing [45,46]. For example, Schneider et al. report the design of peptide, MAX1, with alternating polar and hydrophobic amino acids that, with changes in pH, can be repeatedly switched between an unstructured state and a β-structured gel [*47]. Related to this, several groups report switches between soluble α-helical states and β-sheet fibrils. Notably, Mihara and co-workers demonstrate switches from a small, covalent α-helical coiled-coil dimer (see next section for a description of this protein structure) to amyloid-like fibrils [*18,48–50]; the switching behaviour is altered by varying the hydrophobicity of termini of peptides. In an extension of their earlier work, Zhang and colleagues demonstrate that some of their designs switch state from α-helix to β-structure in response to changes in solvent conditions [51]. Most recently, Ciani et al. describe a temperature-induced switch from an α-helical coiled-coil dimer to β-sheet fibrils [*52]. This design is noteworthy because two designed sequence motifs compatible with each of the target structures—i.e. a dimeric coiled coil and a β-hairpin—are superimposed in the same peptide.

The use of β-sheet fibrils is not restricted, however, to mimicking biological materials or promoting a biological function. Recently, Scheibel et al. [**53] and Reches and Gazit [**54] independently describe templated assembly of metal nanowires using β-sheet fibrils. Scheibel et al. conjugate colloidal gold particles to exposed cysteine residues engineered into the fibril-forming yeast protein Sup35p. This is followed by reductive deposition of silver and gold onto the modified fibrils to give conducting wires ~100 nm in diameter and with a resistance of 86 Ω. In contrast, Reches and Gazit employ a diphenylalanine peptide that forms nanotube structures with the structural and tinctorial properties of amyloid fibrils, a surprising observation given the small size and nature of this peptide. Addition of ionic silver to solutions of these materials results in the deposition of nanoparticulate silver inside the tubes. Reduction of the silver and removal of the templating peptide by proteolysis gives silver nanowires with a consistently uniform diameter of only ~20 nm. The conductivity of these wires is unknown. Using proteins originally recovered from a combinatorial library [55], Hecht and colleagues demonstrate the templated assembly and orientation of amyloid-like fibrils by a highly ordered pyrolytic graphite surface [**56].

Finally, the assembly of β-sheet fibrils is not restricted to canonical polypeptides. Several groups have demonstrated the modification of β-sheet fibrillar scaffolds with non-biological components to give hybrid structures. Kelly and co-workers report the synthesis of a peptidomimetic of two short peptides linked and oriented by a dibenzofuran group, and terminal *N*,*N*-dimethyl ethylenediamine groups to control self-assembly [57]. Despite significant modification of the biological scaffold,

this construct forms amyloid-like fibrils whose morphology can be controlled by pH. Burkoth et al. report the formation of β-sheet fibrils by a hybrid system comprising a portion of the aforementioned Aβ peptide conjugated to PEG-3000 [58]. Maji et al. propose the formation of β-sheet fibrils by tri-peptides comprising the unnatural amino acids β-alanine and α-aminoisobutyric acid, and dipeptides containing 3-aminophenylacetic acid [59,60]. These results further support the hypothesis that it is the ubiquitous polypeptide backbone, rather than side-chain chemistry, that dictates β-sheet fibril assembly, which, in turn, suggests that a wide variety of fibrous materials might be engineered around this structure.

3. α-Helix-based assemblies

Turning to α-helical assemblies: why do we consider these to be better understood than β-sheet assemblies and how might this be of use in materials science? At least until recently, α-helical folding and assembly received more attention from both theoretical and experimental perspectives. This partly reflects the point that the α-helix is a 'self-contained' and true element of secondary structure, whereas β-sheets usually involve non-local (tertiary) interactions, i.e. hydrogen bonding between distant β-strands that are not necessarily in the same polypeptide chain. This has hampered bioinformatics, theoretical and experimental studies of β-sheet folding and stability relative to similar studies of α-helix folding. Nonetheless at present, because of the research activity in amyloid and amyloid-like structures, the development of fibrous-protein materials based on the α-helix lags behind.

One example of a well-studied and well-defined α-helical motif is the coiled coil [61,62]. Most coiled-coil sequences display a repeating pattern of hydrophobic and polar amino acids, $(HPPHPPP)_{n \geqslant 4}$. This directs the folding of amphipathic α-helices, which, in turn, self-assemble into bundles to bury their hydrophobic surfaces. Although the oligomerisation and topologies of coiled coils vary, the packing of the hydrophobic faces is intimate and better defined than in any other type of protein structure. As a consequence and through numerous studies over the last decade, sequence-to-structure relationships have been established for some of these topologies, most notably parallel dimers, trimers and tetramers. This understanding has led to some of the most successful protein designs to date [63,64], and is now forming the basis for the development of new fibrous-protein materials de novo. Furthermore, the α-helical coiled coil seems a sensible starting point for making such structures because it is what nature utilises in many of its fibrous proteins, e.g. the intermediate filaments and certain extracellular matrix proteins [1,2].

To our knowledge, there have been four independent reports of designed α-helical coiled-coil-based fibres. The first is from Kojima et al. [65]. The design, α3, is for a 3-heptad coiled coil, which associates to form water-soluble helix bundles. However, under certain conditions (pH 6, 0.1 M KCl and above) the peptide forms fibres 5–10 nm in diameter, which bundle to form fibres that extend for microns. The secondary structure of these fibres has not been not probed or described in detail. Pandya et al. provide the second report [66]. They describe two 'SAF' peptides designed to combine in an offset manner to give a ''sticky ended' heterodimeric leucine zipper, which promotes longitudinal assembly of the fibres. Curiously, the fibres are thicker than designed indicating that they also assemble laterally. The final, matured structures tend to be tens of microns long and tens of nanometers thick. The appeal of this system compared with others is that it comprises two complementary peptides, which on their own are unfolded and only co-assemble when mixed; this feature raises possibilities for introducing extra levels of control in fibre formation and decoration (see comments on the papers by Ryadnov and Woolfson below). A third example is from Ogihara et al. [67]. In this case, a crystal structure for the building block, which is a 3-helix bundle, is used to guide two redesigns. One of these is for an asymmetric helical hairpin structure that has to form interwined oligomers to complete each 3-helix bundle. This is an example of so-called 3-D protein-domain swapping. The redesigned protein, DSAg, forms fibres consistent with the desired head-to-tail interwined oligomerisation, and ultrastructure observed by electron microscopy is consistent with the designed protofibrils bundling to form thickened fibres 30–70 nm across. Finally, Kajava and co-workers [68] describe fibres produced from a pentameric coiled-coil building block, which is rare in nature. The design principles that ensure longitudinal extension of the coiled-coil building blocks are less clear than in the previous examples, and the fibres are shorter. Nonetheless, the fibres are extremely heat stable and, furthermore, the single-thickness (2.5 nm) fibres produced are fully consistent with the expected width of the designed coiled-coil building blocks.

In two papers last year [**69,*70], Ryadnov and Woolfson extend the earlier studies of Pandya et al. [66]: first [**69], they introduce a second-generation SAF design, Fig. 3a, which gives more stable and better-defined fibres than described by Pandya et al. In addition, this paper proposes and demonstrates a concept for kinking and branching the fibres, which otherwise form exclusively linear structures. Briefly, non-linear 'special' peptides are added to the standard SAF peptides mixtures. The amount of kinking and branching varies with the type and concentration of the special peptide added, and also with the fibre background used; the first-generation fibres are more susceptible to kinking,

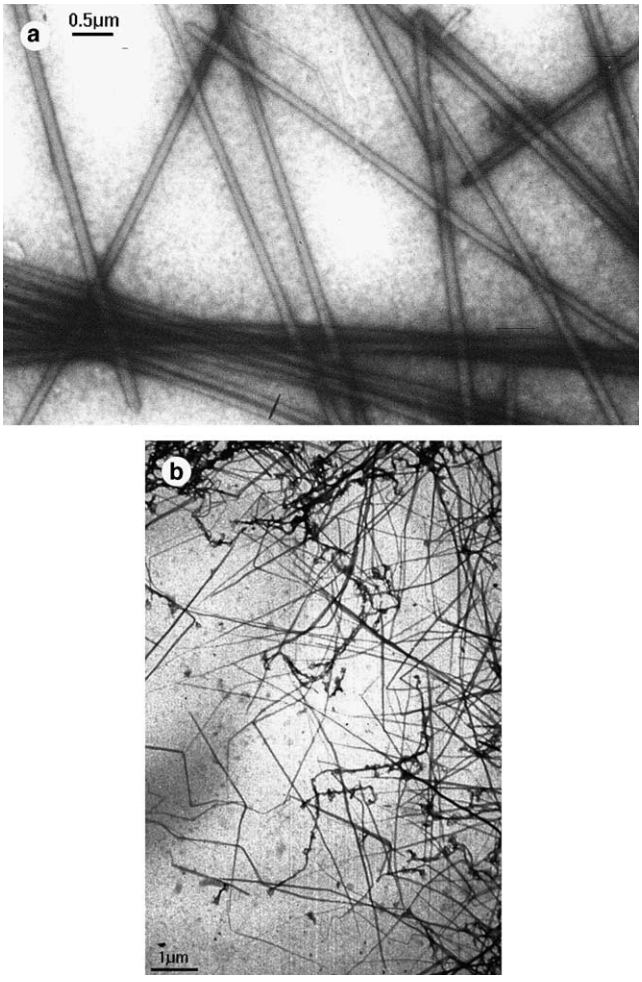

Fig. 3. Transmission electron micrographs of coiled-coil-based self-assembling fibres (SAFs) [66,**69]. (a) The second-generation SAFs [**69], which are better defined and more stable than those previously reported [66]. (b) First-generation SAFs incorporating special peptides designed to kink the fibres [**69].

Fig. 3b. In the second paper [*70], 'T-shaped' special peptides are introduced into the standard SAF mixtures to form branched structures. The bar of the T is a complete standard SAF peptide, whilst the stem is one half of one of the same peptide; this so-called T-SAF can seed fibrillogenesis in three directions. One problem with this approach is that T-SAFs appear not to be completely tolerated by the fibres and their incorporation and the resulting branches are quite rare.

3.1. Engineering fibrous assemblies using natural globular protein domains

Yeates and colleagues offer a third approach to the design of protein fibres [71]. In this case, the building blocks are natural protein domains, which are independently and often robustly folded structures. Specifically, oligomeric proteins, e.g. dimers and trimers, are engineered to create chimeric proteins (also called fusions) that present surfaces to combine specifically and give large assemblies. For instance, a chimera that presents two diametrically opposed dimerisation surfaces is shown to form fibres; more complex examples exist in which dimerisation and trimerisation interfaces are combined in fusions that lead to more complex assemblies. This type of work is reviewed more thoroughly by Yeates and Padilla [**72]. Similarly, Hyman et al. show that one of the protein components, P37, of a fibrous projection from bacteriophage T4 can be engineered whilst maintaining the ability to assemble into fibres [73]. They propose that this provides a basis for nanometer-scale engineering. Finally, though not strictly a fibrous assembly, Moll et al. demonstrate beautifully that a bacterial S-layer protein, which forms 2-D crystals on the cell surface, can be engineered and used as a platform to create nanometer-scale arrays of functional proteins [*74], which further demonstrates the potential for forming functional materials from decorated peptide and protein assemblies.

4. Conclusions

We have attempted to convey the breadth and depth of the growing field of peptide and protein-based biomaterials whilst focusing on engineered and de novo designed fibrous structures built from short, synthetically accessible and experimentally amenable peptides. At the moment, research in this area using amyloid and amlyloid-like fibrils is rich and moving quickly. α-Helix-based fibres, however, are also emerging that offer complementary and alternative routes and possibilities for the rational design and controlled bottom–up assembly of fibrous systems. In this process, we have only glanced upon certain related fields such as the use of larger natural proteins in nanometer-scale engineering, and the development of block co-polymers that contain peptide units and self-assemble into fibrous and nanostructured materials. What we hope to have made clear, however, is that there is tremendous scope in all of these areas: based either on natural or engineered biomolecules directly, or simply by taking inspiration from biological structures, the potential for making new fibrous biomaterials is considerable. The possible applications for such *soft bio-inspired materials* are also wide. This is because the materials self-assemble in water, and many, if not all, aspects of the assembly may be programmed into their chemical structures (i.e. bottom–up assembly). Finally, the fact that such materials will be, or could be made biocompatible opens up possibilities for engineering devices, sensors and materials for medical applications.

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