Controlled Fibril Assembly

Controlled Assembly of Macromolecular β-Sheet Fibrils**

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Construction of functional molecular devices by directed-assembly processes is one of the main challenges in the field of nanotechnology.[1,2] Many approaches to this challenge use biological assembly as a source of inspiration for the build up of new materials with controlled organization at the nanoscale. In particular, the self-assembly properties of β-sheet peptides have been used in the design of supramolecular materials, such as tapes, nanotubes, and fibrils.[3–6]

The incorporation of self-organizing peptides into synthetic polymeric materials has been investigated by a number of groups, all of whom were working on fully synthetic approaches. Lynn and co-workers studied the dilute-solution properties of a diblock copolymer of the central domain of β-amyloid peptide and poly(ethylene glycol) (PEG).[7–9] Rathore and Sogah synthesized multiblock copolymers of oligopeptide β-sheet silk domains and PEG.[10,11] The work of Klok and co-workers is another interesting example as they reported the solid-state/melt properties of block copolymers of PEG and an amphiphilic β strand peptide.[12] The introduction of protein engineering to the field of materials science means that the structural peptide unit can be extended from peptides to tailor-made, monodisperse, high-molecular-weight macromolecules of controlled amino acid sequence.[13–15] The predetermined composition of these proteins leads to a higher level of control over folding than can be achieved with smaller peptide elements. Furthermore,

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the assembly properties of a block copolymer consisting of a designed, genetically engineered, high-molecular-weight polypeptide and PEG have not been described to the best of our knowledge.

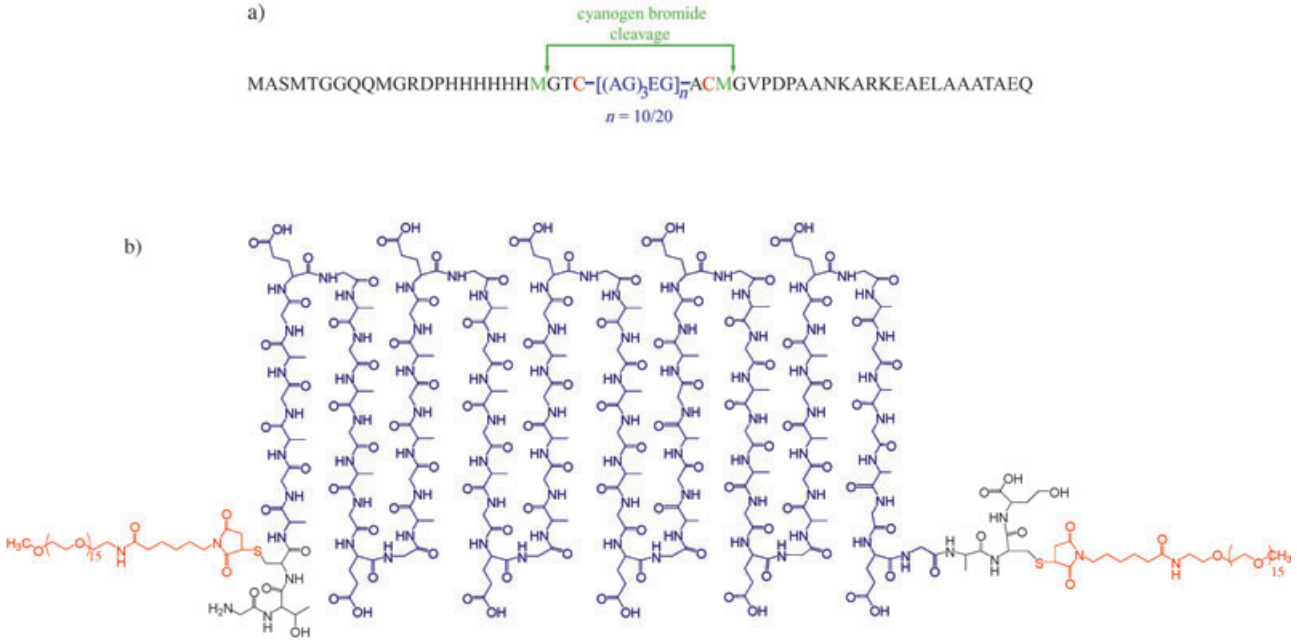
The feasibility of controlling the solid-state structure of polymeric materials through protein engineering was shown by Krejchi et al.[16,17] The observation that alanylglycine (AG) repeating units form extended β strands in a variety of AG-rich polypeptides, including *Bombyx mori* silk fibroin,[18] was the basis for the preparation of polypeptides containing the repetitive sequence $[(AG)_xEG]_n$ ($x = 3$–6). Insertion of the more polar and bulky glutamic acid (E) residue into this sequence resulted in controlled crystallization from formic acid and the formation of needle-shaped lamellar crystals with the glutamic acid residues confined to the crystal surface. Although the folding pattern of the polypeptide chain was well controlled, assembly resulted in extended-plate-like structures. Therefore, some of the information present in the polypeptide design, such as the height and width of the β-sheet elements, was lost during the aggregation process.

Herein, we describe the preparation and assembly of a triblock copolymer consisting of a central β-sheet polypeptide block composed of the repetitive $[(AG)_3EG]_n$ sequence conjugated to PEG end blocks. The rationale behind the attachment of synthetic polymer blocks at the N and C termini was to restrict macroscopic crystallization and allow translation of the β-sheet design characteristics of width, height, and surface functionality into the self-assembled structures.

We first constructed $[(AG)_3EG]_n$ β-sheet polypeptides outfitted with N- and C-terminal cysteine residues by protein engineering. The target sequence of this protein is depicted in Scheme 1a. The presence of a hexahistidine tag enabled purification of the polypeptides from the *Escherichia coli* cell lysates by nickel–nitrilotriacetate (Ni–NTA) affinity chromatography. The thiol functionality of the cysteines was subsequently used for selective alkylation with maleimide-functionalized PEG of $M_n = 750\ g\,mol^{-1}$ (Scheme 1b). Conjugates were prepared by reaction with an excess of maleimide-functionalized PEG, followed by removal of this excess by a second Ni–NTA affinity chromatography step. Furthermore, cyanogen bromide (CNBr) cleavage allowed removal of the residual N- and C-terminal amino acids introduced during the cloning process. The preparation of DNA constructs, protein expression/purification, conjugation reactions, and final characterization by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis, ^1H NMR spectroscopic analysis, and MALDI-TOF mass-spectrometric analysis is described in the Supporting Information.

Crystallization experiments were performed both with conjugates still containing the N- and C-terminal amino acids and with conjugates that had been cleaved with CNBr. Crystallization was induced by vapor diffusion of methanol into a solution ($10\ mg\,mL^{-1}$) of the protein in 70% formic acid and resulted, in all cases, in the formation of transparent gels. Attenuated total-reflectance IR and circular dichroism spectroscopic analysis of the conjugates indicated that the antiparallel β-sheet architecture was retained upon conjugation with PEG (see the Supporting Information).

The assembly behavior of the crystallized conjugates was analyzed by transmission electron microscopy (TEM) with samples deposited on carbon-coated grids. This was achieved by either placing the grid with the carbon side down on top of the gel or by diluting the gel tenfold in methanol and then placing the grid on top of this suspension. Both approaches resulted in the same morphology. The TEM grid was covered consistently with fibrillar structures for PEG–$[(AG)_3EG]_{20}$–PEG conjugates. Aligned micrometer-long fibrils with a width of approximately 12 nm were observed for the variant still containing the nonrepetitive amino acids (Figure 1a). A



Scheme 1. a) Target protein sequence; b) ABA-type block copolymer formed by reaction of maleimide-functionalized PEG ($M_n = 750\ g\,mol^{-1}$) with the cysteine-flanked $[(AG)_3EG]_n$ β-sheet element.

network of individual fibrils with the same width was observed when the residual amino acids were cleaved from the conjugate (Figure 1b). Only a few individual aggregated fibrils were found on the grid surface for the proteins without

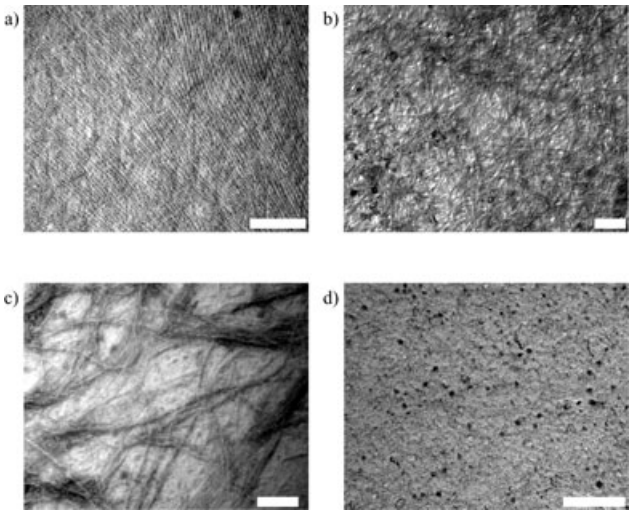


Figure 1. Transmission electron micrographs of crystallized PEG–$[(AG)_3EG]_n$–PEG conjugates: a) PEG–$[(AG)_3EG]_{20}$–PEG before CNBr cleavage; b) PEG–$[(AG)_3EG]_{20}$–PEG after CNBr cleavage; c) PEG–$[(AG)_3EG]_{10}$–PEG before CNBr cleavage; d) PEG–$[(AG)_3EG]_{10}$–PEG after CNBr cleavage. The scale bars represent 200 nm.

conjugated PEG (see the Supporting Information); this effect is most probably the result of the formation of larger aggregated structures because of the increased interaction between the β-sheet polypeptides.

Comparison of this texture with PEG–$[(AG)_3EG]_{10}$–PEG conjugates, in which the β-sheet block was exactly halved, also showed the formation of fibrils for the uncleaved PEG–$[(AG)_3EG]_{10}$–PEG sample (Figure 1c). However, the fibrillar width was clearly smaller than that of the PEG–$[(AG)_3EG]_{20}$–PEG conjugates as it could not be measured because, in this case, the grain size of the platinum particles was too large relative to the fibril width to distinguish single fibrils. Single fibrils were difficult to distinguish because of dense coverage of the grid for the CNBr-cleaved PEG–$[(AG)_3EG]_{10}$–PEG conjugate (Figure 1d).

To investigate further the fibrillar texture and the effect of β-sheet length, we analyzed the fibril dimensions by atomic force microscopy (AFM). Most importantly, the AFM experiments proved the presence of fibrils for samples spincoated on mica surfaces, although no single fibrils could be observed for the CNBr-cleaved PEG–$[(AG)_3EG]_{10}$–PEG conjugate by using TEM (Figure 2a). Individual fibrils were also visualized for the CNBr-cleaved PEG–$[(AG)_3EG]_{20}$–PEG conjugate, the result of which indicates that the fibrils are stable upon dilution (Figure 2b). Furthermore, fibril heights of approximately 2 nm were measured for PEG conjugates of both $[(AG)_3EG]_{10}$ and $[(AG)_3EG]_{20}$.

An organization in which the hydrogen-bond direction is perpendicular to the fibril direction is plausible when these data are taken into consideration. Fibril formation in the β-

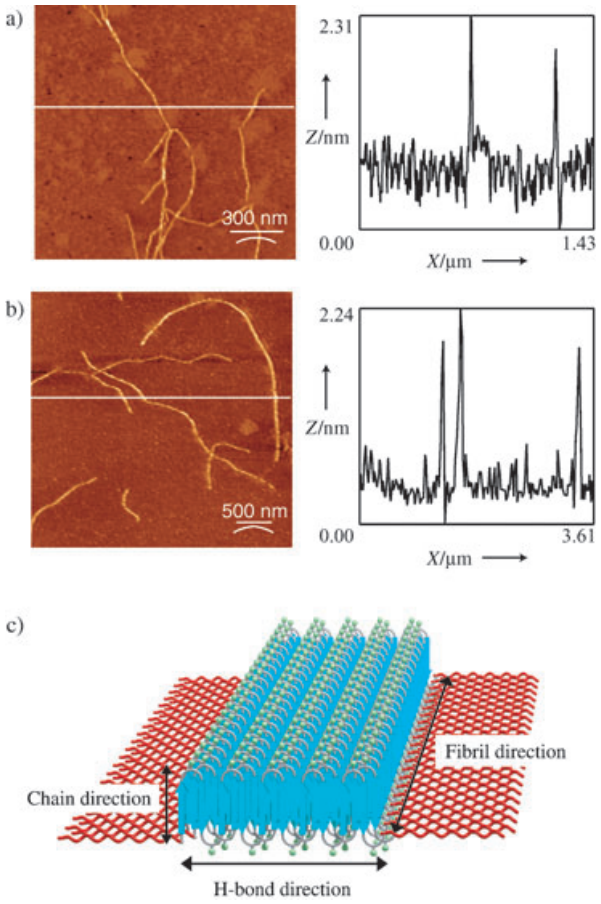


Figure 2. Tapping-mode AFM images of β-sheet fibrils on a mica support: Height image and height profile from single fibrils of a) PEG–$[(AG)_3EG]_{10}$–PEG and b) PEG–$[(AG)_3EG]_{20}$–PEG conjugates. c) Schematic illustration of the proposed organization within the β-sheet fibrils.

sheet stacking direction then results in an assembly in which the hydrophobic β-sheet surfaces are shielded from the polar solvent, whereas the PEG chains are in contact with the environment. This organization is schematically depicted in Figure 2c. The height values of approximately 2 nm measured for both conjugates are supportive for this type of organization. These fibril heights are in reasonable agreement with the calculated height of 2.8 nm, particularly when one takes into account the fact that AFM measurements do not give absolute height values as a result of possible deformation caused by the tip's pressure and the influence of air humidity.[19] The wide-angle X-ray diffraction data from Krejchi et al.[17] shows that the lengths of the crystalline blocks in the hydrogen-bond direction are 4.3 and 8.9 nm for the $[(AG)_3EG]_{10}$ and $[(AG)_3EG]_{20}$ conjugates, respectively. An accurate indication of the β-sheet width of the PEG–$[(AG)_3EG]_{10}$–PEG and PEG–$[(AG)_3EG]_{20}$–PEG conjugates proved to be difficult because of the geometry of the probe tip (it typically has a finite size of 10 nm). A qualitative assessment of the combination of AFM and TEM data, however, showed that the PEG–$[(AG)_3EG]_{20}$–PEG fibrils were consistently broader than the PEG–$[(AG)_3EG]_{10}$–PEG assemblies, whereas no difference in height was observed. X-ray

measurements were carried out to obtain conclusive evidence for the crystalline organization within the fibrils and the direction of the hydrogen-bonding and hydrophobic interactions, but this turned out not to be trivial and needs to be explored in more detail.

In conclusion, we have shown that the attachment of PEG end blocks to a central poly[$(AG)_3EG$] β-sheet block prevents the macroscopic aggregation of the β-sheet blocks into needle-shaped lamellar crystals. Instead, crystallization results in well-defined fibrils. Microscopy data suggest an organization in which fibrils are formed in the β-sheet stacking direction. Furthermore, absolute control over the amino acid sequence might offer the possibility of introducing specific amino acid residues at the turns of the β-sheets, thereby creating a regular array of functional moieties at the fibril surface. Patterning of the surface with different amino acid combinations might provide a method for positioning different chemical functionalities on the nanometer scale.

Experimental Section

TEM: All samples were prepared on copper specimen grids covered with a carbon support film (CF200-Cu; Electron Microscopy Sciences, Washington, USA). The grids were placed with the carbon film down on top of a 5-μL droplet of protein suspension (1 mg mL^{-1}) in methanol or on a gel (10 mg mL^{-1}) for 30 s. The grids were allowed to dry and were subsequently shadowed with platinum at an elevation angle of 45°. Images were recorded by using a JEOL 1010 electron microscope.

AFM: Protein solutions with concentrations of 10–100 μg mL^{-1} were spincoated onto a freshly cleaved mica surface. The morphologies of the samples were analyzed by means of tapping-mode AFM with a Nanoscope IIIa instrument operating in air at room temperature. Height and phase images were recorded with microfabricated silicon cantilevers (length of 100 mm and width of 35 mm) that had a spring constant of 5.5–22.5 N m^{-1} by using scan rates of 1–2 lines s^{-1} and a resolution of 512 × 512 pixels.

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