FORMATION OF POLYMER NANOFIBERS FACILITATED BY THE SELF-ASSEMBLY OF TEMPLATE PRE-ORGANIZED OLIGOPEPTIDES

Hans G. Börner[1] and Doreen Eckhardt[1]*

(1) Max Planck Institute of Colloids and Interfaces
Colloid Department, 14424 Potsdam, Germany
hans.boerner@mpikg-golm.mpg.de

Introduction

Many structure formation processes within biomaterials are guided by the self-organization of polypeptides or proteins.[1] Utilizing these organization principles for the structuring of synthetic materials would be of great interest.[2-4] Particularly the potential of the β-sheet motif to form nanostructured fibrillae or fibers is highly attractive and therefore it has been investigated intensively.[5-8] Burkoth *et al.* pioneered this work by the investigation of a conjugate comprising of poly(ethylene oxide) (PEO) and the $A\beta_{10-30}$ fragment of the amyloid sequence. It was shown that soluble fibrillar aggregates could be obtained, allowing the investigation of amyloidal structures in aqueous solution.[9]

The rational design of robust fiber-like PEO structures exploiting the self-assembly of oligopeptide-organizer units requires peptides with high tendencies to form stable β-sheets. Thus, a pre-organization strategy was applied. The attachment of oligopeptides to a novel template pre-organizes the strands into optimized geometry and restricts their conformational freedom. This results in a strong enhancement of the formation of anti-parallel β-sheets.[10]

Experimental

Materials and Instrumentation: Materials and procedures used for the preparation of the template, the peptides and the conjugate have been described elsewhere.[11] Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems Inc.). Circular dichroism UV spectra were recorded in solution on a Jasco 715 CD spectrometer. Atomic force microscopy (AFM) was performed on a NanoScope IIIa device (Veeco Instruments) in tapping mode.

Results and Discussion

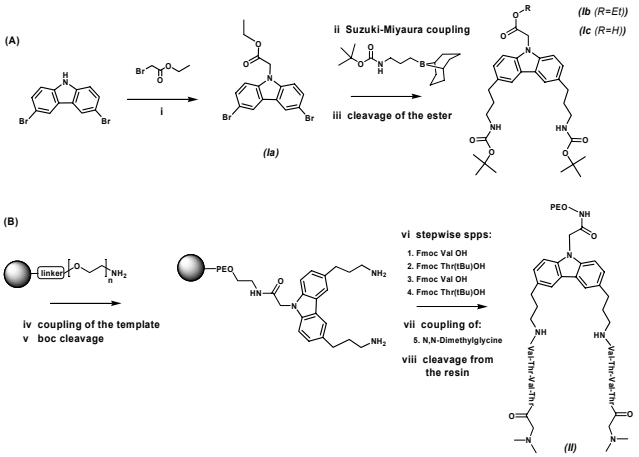
Stimulated by a dibenzofuran-2,8-dipropanoic acid template[8] designed by *Kelly et al.*, a novel 3,6-bis(3-aminopropyl)-carbazole-9-acetic acid (***I***) was synthesized (Scheme 1A).

The template exhibits two functionalities required for the tethering of the two oligopeptide strands (Scheme 1A) and a third one that is orthogonally addressable. The latter allows the attachment of the synthetic polymer chain. Furthermore, it facilitates the conjugation of the oligopeptide strands towards the template since a direct solid-phase supported peptide synthesis can be utilized.

Synthesis of the Organizer-polymer conjugate: Template ***Ic*** is conveniently accessible from 3,6-dibromo-carbazole (Scheme 1A) in a three step synthesis in about 45% isolated yield. The intermediate products ***Ia***, ***Ib*** and the final ***Ic*** gave conclusive ^{1}H-, ^{13}C-NMR. Furthermore, the chemical structure of ***Ib*** was confirmed by MS analysis.

To access the conjugate ***II***, composed of PEO and the template attached oligopeptides, a solid-phase supported strategy was selected (Scheme 1B). The application of a Tentagel® PAP resin ($DP_{n,\,PEO} \approx 73$) allows the direct synthesis of PEO-oligopeptide conjugates.[12] After resin attachment of ***Ic***, the oligopeptide strands can be subsequently synthesized, following standard Fmoc-protocols.[13]

The sequence of alternating threonine and valine ($(Thr\text{-}Val)_2$) residues was chosen because both amino acids have high β-sheet propensities and the hydrophobic-hydrophilic repeat pattern matches the β-strand periodicity.[14] The N-terminal capping of the template tethered peptides with N,N-dimethylglycine (DMG) generates cationic strand termini depending on the pH-value. These enhance the solubility and contribute to the formation of anti-parallel β-sheets.[8]



Scheme 1: ***(A)*** Preparation of the amin protected template (i K_2CO_3, DMF, 55°C; ii: Suzuki-Miyaura cross-coupling: aq. KOH/toluene, $[Pd(PPh_3)_4]$, 110°C; iii: KOH/THF, 60°C); ***(B)*** Synthesis of the PEO-template(oligopeptide)$_2$ conjugate (iv: PyAOP, DIPEA, NMP; v: TFA/DCM; vi: HBTU, DIPEA, NMP; Piperidine/NMP; vii: PyBOP, DIPEA, DMF; viii: TFA/ TMSBr).

The complete conjugate ***II*** is obtained in about 45% isolated yield after liberation from the support, re-precipitation and dialysis. The molecular structure was confirmed by MALDI-TOF-MS analysis (Figure 1). The mass peaks could be assigned to the homologues series of ***II*** with an average reminder mass of about ±0.5 Da.

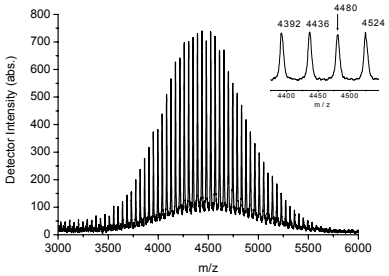


Fig. 1: MALDI-TOF-MS spectrum of ***II***

Aggregation behavior: The aggregation behavior of ***II*** was investigated by applying a de-aggregation aggregation process.

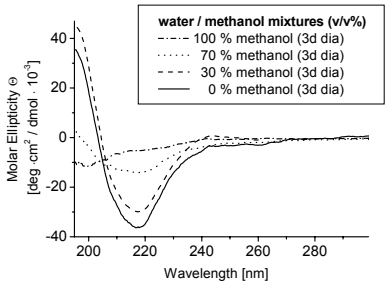


Fig. 2: UV-CD spectra of 1 mg/mL solutions of ***II*** in different compositions of the solvent.

Complete de-aggregation and denaturation of the secondary structure was achieved by treatment with trifluoroacetic acid (TFA). This was confirmed by circular dichroism UV spectroscopy (CD) indicating mainly a statistic segment configuration of the peptide strands (minimum 197 nm). Furthermore, with AFM no obvious formation of ordered structures could be detected (data not shown). After multiple cycles of dilution with methanol and concentration by vacuum distillation, the polymer solution was dialyzed against pure methanol. The subsequent stepwise exchange of methanol with water leads to a progressive increase of the fraction of β-sheet secondary

structures as it could be shown by CD (Fig. 2). The characteristic Cotton effects (minimum at 218 nm and the maximum at 198 nm) that indicate peptide strands adapting the β-sheet motif can be clearly observed.[15] Along with the evolving β-sheet motifs, extended fiber-like structures were visualized in the AFM micrograph (Fig. 3).

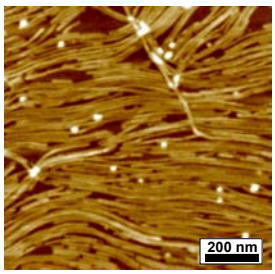


Fig. 3: PEO fiber aggregates of ***II*** after dialysis. AFM micrograph (0.5 mg/mL aq. solution, spin coated on Mica, tapping mode)

The fibers exhibit a number average height of about 1.4 ±0.1 nm and a maximum length in the range of 1 µm. In a close parallel packing of fibers observed in the AFM micrograph an average lateral spacing of about 13.6 ±1 nm was measured between the height maxima of the fibers (Fig. 4). These spacings might provide a hint to the width of the solution structure. That would meet the expected theoretical width of approximately 12-16 nm if a random coil PEO chain and a typical β-strand pitch of 3.5 Å per amino acid is assumed.

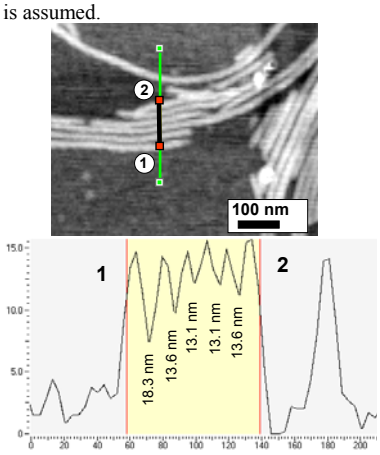


Fig. 4: left: AFM micrograph of ***II***; 0.08 mg/mL aq. solution, pH ≈ 7, spin coated on Mica, tapping mode (z = 8 nm height); right: height profile of parallel fibers (cross section analysis)

Aggregation model: Based on the observations presented above as well as on literature discribing the aggregation behavior of pre-organized oligopeptides,[8] a preliminary model can be suggested.

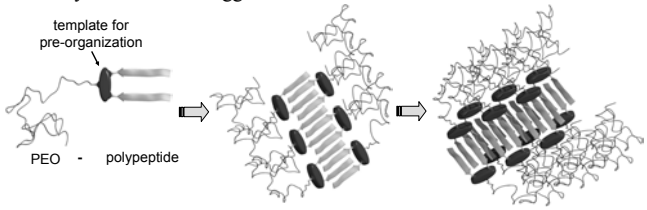


Fig. 5: Idealized structure proposal for the aggregation of ***II*** into fiber like aggregates showing a peptide core and a PEO shell

As outlined in figure 5, the final fiber structure is most likely stabilized by the formation of an anti-parallel β-sheet of the oligopeptide units. Thus the aggregate probably exhibits a core-shell structure comprising of an oligopeptide β-sheet core and a PEO shell. The fiber structures are probably composed of double β-sheets denoted as ribbons. This assumption is supported by the average height of the fibers of about 14 Å. A ribbon usually exhibits a 9-12 Å inter sheet spacing.

pH-responsive organizer units: By adapting the sequence of the pre-organized oligopeptides, organizer units are obtained that exhibit a pH-window responsive aggregation behavior. Therefore, amino acids with ionizable side groups were incorporated at the termini of the peptide strands (Figure 6). Depending on the pH-value oppositely charged groups are generated that contribute via ion pair interactions to the stability of the antiparallel β-sheet (Figure 6).

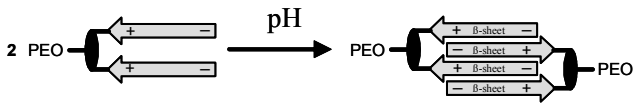


Fig. 6: Idealized structure proposal for the aggregation of PEO-template(RAGAE-Ac)$_2$ stabilized by ion pair interactions

As peptide sequence Arg-[Ala-Gly-Ala]-Glu (RAGAE) was chosen. The side groups of arginine and glutamic acid are ionized within a wide pH-range, stabilizing the aggregation of the alanine-glycine-alanine segment that is a typical sequence known to form β-sheets (e.g. in spider silk). However, without ion pair bonding AGA is too short and not capable of forming a stable β-sheet. Thus, a reversible switching of the aggregation and the de-aggregation can be induced via changing the pH-value. Preliminary studies reveal the reversible formation of β-sheet structures (CD) as well as the formation of fiber-like nanostructures (AFM analysis) (not shown).

Conclusion: The attachment of two short peptides to a novel template results in an optimized pre-organization of the strands in order to form antiparallel β-sheet motifs. These rationally designed peptide organizer units were conjugated to poly(ethylene oxide) yielding macromolecular LEGO® bricks that inherently contain the information required for the desired structure. Via self-assembly of the organizer units, structure could be induced within the synthetic polymer. As a result fiber-like nanostructures were obtained exhibiting a defined peptide core and a poly(ethylene oxide) shell. The tendency of the organizer units to form defined structures can be modulated over a broad range. It was demonstrated that a change in the peptide sequence makes pH-responsive organizers accessible.

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