Rational design of oligopeptide organizers for the formation of poly(ethylene oxide) nanofibers†

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Template pre-organized oligopeptides were conjugated to poly(ethylene oxide) chains yielding peptide-polymer-building blocks that self assemble into fiber-like nanostructures having a maximum length in the range of a micrometer.

Numerous native construction-materials exhibit well-adapted, high performance properties due to their optimized structural design.[1] Frequently, the structure formation processes within these biomaterials are guided *via* self-organization of polypeptides or proteins.[2] Transferring these organization principles towards the structuring of synthetic materials would be of great interest. Some possibilities resulting from peptide-guided structure formation within synthetic polymers have been recently demonstrated.[3]

Important structural elements in native materials are fibrils or fibers. Diverse properties like anisotropic strength,[4] structural stability[5] or directed transport[5] can be realized with these structures. In material science, nanofibers are of high interest due to their potential applications, as high-strength components in composite materials,[6] nanowires,[7] fibers for medical applications[8] or macromolecular actuators.[9] Therefore, preparation of polymer nanofibers *via* a peptide-guided route would be innovative. Sequence controlled oligopeptides that self-assemble spontaneously, or even triggered, into nanostructured fibrillae or fibers have been investigated intensively.[10–12] Although the principles for peptide design are only roughly understood, a tendency to form β-sheet motifs appears to be essential to realize self-assembly into fiber structures.[11]

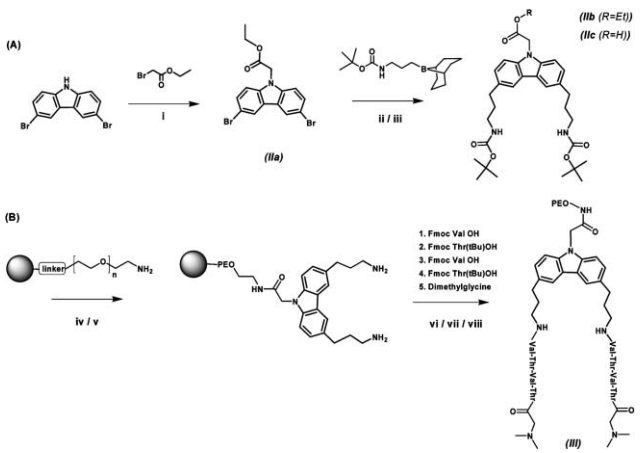
Burkoth *et al*. utilized a conjugate of poly(ethylene oxide) (PEO) with the $A\beta_{10-30}$ fragment of the amyloid sequence to achieve soluble amyloidal aggregates, allowing the investigation of such structures in aqueous solution.[13] Recently, Messersmith *et al*. demonstrated that also shorter oligopeptides (*e.g*. 11-mers) have the inherent potential to self-organize PEO into soluble fibers due to suppression of lateral fiber aggregation.[14]

However, 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. To design such organizers we have been inspired by the work of Kelly *et al*. who investigated the β-sheet formation of template pre-organized oligopeptides.[12] It has been shown that the restriction of the conformational freedom of oligopeptide strands and the pre-organization into optimized geometry strongly enhances the formation of anti-parallel β-sheets.[15] Thus, herein we investigated template pre-organized oligopeptides as organizing units for the preparation of polymeric, fiber-like nanostructures.

Stimulated by the dibenzofuran-2,8-dipropanoic acid template[12] (***I***),‡ a novel 3,6-bis(3-aminopropyl)carbazole-9-acetic acid (**II**) has been designed (Scheme 1A). Compared to **I**, a third functionality is present in addition to those required for the tethering of the two oligopeptide strands (Scheme 1A). The new functionality can be orthogonally addressed and allows the attachment of a synthetic polymer chain. Furthermore, it facilitates the conjugation of the oligopeptide strands towards the template. Instead of the necessary solution-phase fragment ligation that is required for coupling oligopeptides to **I**,[12] in the case of **II** a direct solid-phase supported peptide synthesis can be utilized.

The template **II** is conveniently accessible starting from 3,6-dibromocarbazole (Scheme 1A). In the first reaction step, α-bromoethyl acetate reacts with the carbazole derivative yielding **IIa** in a yield of about 84%. The subsequent Suzuki–Miyaura cross-coupling of **IIa** with a hydroborated *N*-(*tert*-butoxycarbonyl)allylamine, followed by hydrolytic saponification, results in **IIc** in about 45% isolated yield. The intermediate products **IIa**, **IIb** and the final **IIc** gave conclusive ^{1}H- and ^{13}C-NMR spectra.† Furthermore the chemical structure of the precursor **IIb** was confirmed by MS analysis.†

To access the conjugate composed of PEO and the template-attached oligopeptides (**III**), a straightforward solid-phase



Scheme 1 Preparation of the amine protected template (A) (i: K_2CO_3, DMF, 55 °C; ii: aq. KOH, toluene, $[Pd(PPh_3)_4]$, 110 °C, 22 h; iii: aq. KOH, THF, 60 °C, 23 h); synthesis of the PEO-(template-oligopeptide) conjugate (B) (iv: PyAOP, DIPEA, NMP, 4 h; v: TFA–DCM (30 v/v%), 30 min; vi: HBTU, DIPEA, NMP, 2 h; piperidine/NMP (20 v/v%); vii: PyBOP, DIPEA, DMF, 2 h; viii: TFA 99%, 1% TMSBr, 2–6 h).

† Electronic supplementary information (ESI) available: materials, synthesis procedures and characterization data for the compounds **IIa–c**; **III** and **IV**. See http://www.rsc.org/suppdata/cc/b5/b503275j/

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supported strategy was selected (Scheme 1B). The application of a Tentagel® PAP resin comprising a cleavable PEO spacer ($DP_{n,PEO} \approx 73$), allows the direct synthesis of PEO-oligopeptide conjugates.[16] After resin attachment of **IIc** following enforced-coupling protocols, the *tert*-butoxycarbonyl (*t*Boc) protecting groups are removed and the oligopeptide strands can be subsequently synthesized. Therefore, automated stepwise amino acid attachment was applied, following standard Fmoc-protocols.[17] Quantitative conversion for each coupling step was confirmed by performing Kaiser tests. In the case of an incomplete reaction, additional amino acid coupling cycles were performed until full conversion.

The primary structure of the oligopeptide strands comprises a diad of alternating threonine and valine [(Thr-Val)$_2$]. This sequence was chosen since (Thr-Val)$_x$-segments have high β-sheet propensities since the hydrophobic–hydrophilic repeat pattern matches the β-strand periodicity.[18] However, in oligopeptides 5–6 repeats of (Thr-Val) are needed to form stable aggregates in water.[18]

By means of N-terminal capping of the template-tethered peptides with *N*,*N*-dimethylglycine (DMG), strand-termini were introduced that exhibit cationic charges depending on the pH-value. These will enhance the solubility and contribute to the formation of anti-parallel β-sheets.[12] The complete conjugate (**III**) is obtained in about 45% isolated yield after liberation from the support, re-precipitation and dialysis (MWCO ≈ 1000). The molecular structure was confirmed by MALDI-TOF-MS analysis.† In addition to **III**, a linear analog (**IV**)‡ was synthesized utilizing a comparable PAP/Fmoc-strategy. The chemical structure of **IV** was DMG-(Thr-Val)$_4$-Gly-NH-PEO$_{68}$ as verified by MALDI-TOF-MS analysis.†

The comparative aggregation study of **III** and **IV** may demonstrate the advantage of the template pre-organization of oligopeptides *versus* linear, non-templated systems. Therefore, the aggregation behavior of **III** and **IV** was investigated by applying a de-aggregation–aggregation route. Complete de-aggregation was achieved by treatment with trifluoroacetic acid (TFA), centrifuging, followed by decanting and partial removal of the TFA in vacuum. After multiple cycles of dilution with methanol and concentration by vacuum distillation, the polymer solutions were dialyzed against pure methanol (MWCO ≈ 1000). The fiber formation process was observed *via* visualization with atomic force microscopy (AFM). Parallel to this the evolution of the secondary structure could be determined by circular dichroism UV spectroscopy (CD).

Denaturation of **III** was confirmed by CD of the methanolic solution. The absence of characteristic cotton effects for β-sheets (minimum at 218 nm and maximum at 198 nm) as well as the occurrence of a minimum near 200 nm indicates that a predominantly unstructured chain configuration of the oligopeptides is present (Fig. 1).[19] In addition to this successful de-aggregation was confirmed by AFM analysis where no obvious formation of ordered structures could be detected.† The stepwise exchange of methanol with water, however, leads to a progressive increase in the fraction of β-sheet secondary structure. This was verified by CD measurements showing an increasing intensity of the characteristic β-sheet absorption bands (Fig. 1).§ Along with the evolving β-sheet motifs, extended fiber-like structures were observed in the AFM micrograph (Fig. 2, left).

The fibers exhibit a number average height of about 1.4 ± 0.1 nm and a maximum length in the range of a micrometer. In a close parallel packing of fibers observed in the AFM micrograph (Fig. 2, left) an average lateral spacing of about 13.6 ± 1 nm was measured between the height maxima of the fibers.† This is in close agreement with the transmission electron microscopy (TEM) image exhibiting a dominant lateral distance between parallel running structures of about 13–17 nm (Fig. 2, right). These spacings might hint to the width of the solution structure that could be calculated, with a value of approximately 12–16 nm if a random coil PEO chain and a typical β-strand pitch of 3.5 Å per amino acid[20] is assumed.

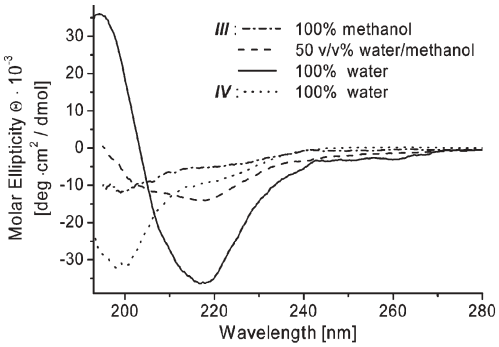

Fig. 1 Comparative UV-CD spectra of 1 mg mL^{-1} solutions of **III** and **IV** (pH-value of all solutions is ≈7).

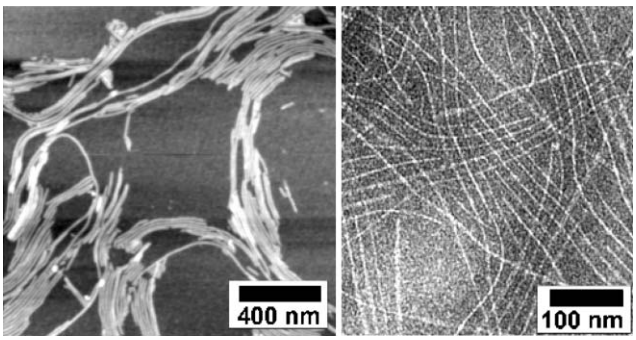

Fig. 2 PEO fiber aggregates of **III**. AFM micrograph (0.08 mg mL^{-1} aq. solution, pH ≈ 7, spin coated on mica, tapping mode, z = 8 nm height) (left) and TEM image (20 mg mL^{-1} aq. solution, negatively stained) (right).

Within the TEM image, discriminative staining causes deviations between the apparent mean width of the observed structures and the lateral spacing. Since negative staining with uranyl acetate was used, PEO might exhibit a more favorable, multidentate uranyl complexation than the rather hydrophobic peptidic structures. This would explain the unstained structures of about 4 ± 3 nm assignable to the peptide parts and the quite uniform lateral spacing between those resulting from surface adsorbed PEO chains. High resolution AFM measurements are currently in progress to support this model. Furthermore, investigations using scattering techniques will allow the determination of more accurate solution as well as solid-state structure dimensions.

Comparable fiber structures with similar dimensions were observed with AFM on mica substrates as well as with TEM on carbon coated copper grids. This indicates that fiber formation is probably not induced by surface interactions since both surfaces have strongly differing surface energies.

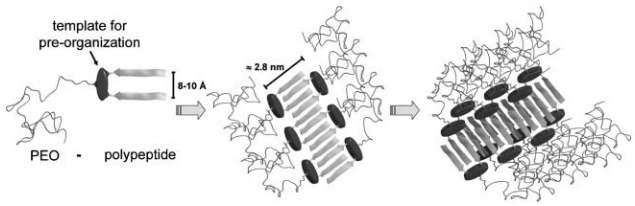

Fig. 3 Idealised structure proposal for the aggregation of **III** into fiber like aggregates showing a peptide core and a PEO shell (peptide structure is displayed enlarged for clarity reasons).¶

Based on the observations described above, as well as on literature describing the aggregation behavior of pre-organized oligopeptides,[12] a preliminary model can be suggested.

As outlined in Fig. 3, 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 an oligopeptide β-sheet core and a PEO shell. The latter contributes to solubility of the fiber and suppresses lateral aggregation. Probably the fiber structures are composed of double β-sheets denoted as ribbons (Fig. 3, right). The ribbon formation might be driven by the hydrophobic effect in order to minimize energy of the hydrophobic valine face of the β-sheets. This assumption is supported by the average height of the fibers of about 14 Å. Since a ribbon usually exhibits a 9–12 Å sheet–sheet spacing, it might be within the range of experimental error.

The visualized fibers exhibit a relatively stiff appearance indicated by the nematic-like order as well as by the absence of obvious mean curvature. This behavior can be explained by the rigidity of extended β-sheets and is thus consistent with the model. The conformational freedom of β-sheet structures is mainly restricted by the nature of hydrogen bonding that stabilizes the structure. Since the binding energy contribution of H-bonding is strongly sensitive towards torsion and distance only limited flexibility of the structures is tolerated.

A comparable treatment of the linear analog **IV**, resulting in de-aggregation and slow transfer into water, does not yield obvious amounts of β-sheet structures. This was shown by CD measurements that indicate a dominant fraction of unstructured oligopeptide chain segments (Fig. 1). Furthermore, with AFM analysis on different substrates no obvious structures could be visualized (data not shown). The absence of defined structures is consistent with the literature[18] and underlines clearly the superior properties of template pre-organized oligopeptides as organizer units.

In conclusion, a synthetic polymer [poly(ethylene oxide)] was organized into nanofibers having a maximum length of up to a micrometer. The biomimetic organization process was guided by oligopeptide self-assembly. Therefore, rational designed oligopeptide-units were synthesized exhibiting an enhanced tendency to form β-sheet motifs. This could be achieved *via* attachment of two rather short oligopeptides to a new template, resulting in an optimized pre-organization of the strands. By comparison of the pre-organized conjugate with a linear PEO-*block*-oligopeptide analog it was demonstrated that pre-organization strongly enhances the aggregation behavior. The tendency of such oligopeptide organizer units to form defined structures can be potentially modulated over a broad range adverting tailor made aggregators with tunable aggregation strength and responsiveness as well as different target structures. The concept of oligopeptide-guided structure formation is currently being widened towards different synthetic polymers to target structured construction materials with tunable mechanical properties.

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Notes and references

‡ Detailed chemical structure is shown in the ESI.†
§ CD band intensities increase consistently during 10%-stepwise solvent exchange (data not shown).
¶Dimensions have been accessed from geometry optimization studies using HyperChem 7.5.[21]

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