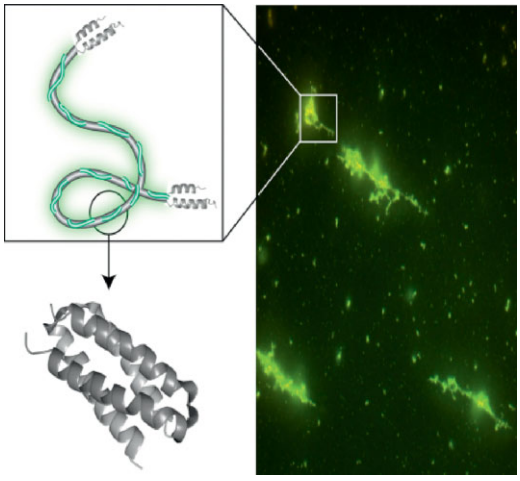
Supramolecular Assembly of Designed α-Helical Polypeptide-Based Nanostructures and Luminescent Conjugated Polyelectrolytes[a]

Jens Wigenius, Per Björk, Mahiar Hamedi, Daniel Aili*

Designed polypeptides with controllable folding properties are utilized as supramolecular templates for fabrication of ordered nanoscale molecular and fibrous assemblies of LCPs. The properties of the LCPs as well as the three dimensional conformation of the polypeptide-scaffold determine how the polymers are arranged in the supramolecular construct, which highly affects the properties of the hybrid material. The ability to control the polypeptide conformation and assembly into fibers provides a promising route for tuning the optical properties of LCPs and for fabrication of complex functional supramolecules with well defined structural properties.



Introduction

The rapid development in the field of conducting conjugated polymers (CPs) is now allowing for the addition of electro-optical properties to biomolecular nano-templates, through self-assembly.[1–3] Biological macromolecules and proteins in particular, offer a rich plethora of possible geometrical and functional templates and scaffolds for applications in bottom-up design of organic-based electronics.[4] Proteins are a chemically and structurally very diverse and versatile group of biomolecules and play a pivotal role as structural scaffolds and highly complex functional units in biological systems. Recently, the possibility to form molecular wires by self-assembly of conducting CPs on protein-based fibers was demonstrated using a polymer with strong affinity for amyloid fibrils.[2] The association of luminescent CPs (LCPs) to various peptides and proteins has further been demonstrated to enable optical detection of different conformational states of biomolecules, including the process of protein misfolding and formation of amyloid fibers.[5,6] The electronic structure, and hence the optical characteristics, of LCPs is strongly influenced by the conformation and organization of the polymer, which is in turn highly dependent on the mode of interaction and the three-dimensional structure of the biomolecular scaffold. Controlling the conformation and oligomerization state of the scaffold thus provide

D. Aili
Division of Molecular Physics, Department of Physics, Chemistry and Biology, Linköping University, SE-581 83 Linköping, Sweden
E-mail: danai@ifm.liu.se
J. Wigenius, P. Björk, M. Hamedi
Division of Biomolecular and Organic Electronics, Department of Physics, Chemistry and Biology, Linköping University, SE-581 83 Linköping, Sweden
D. Aili
Current address: Department of Materials and Institute of Biomedical Engineering, Imperial College London, SW7 2AZ London, UK

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means of controlling the formation of the supramolecular complex and allows for tuning of the optical properties of the LCPs and for self-assembly of advanced molecular nanostructures.

In this communication we report on the folding-dependent association of de novo designed helix-loop-helix polypeptides with controllable folding properties and luminescent conjugated polyelectrolytes. The polypeptides fold into four-helix bundles as a result of heterodimerization and have further been modified to associate into nanofibers as a result of a folding and pH-dependent hetero-association.[7] Designed polypeptides are chemically and structurally very robust molecular entities that can be engineered with high precision and enable incorporation of functional moieties and non-natural amino acids, thus presenting a versatile molecular tool-box for nanoscale self-assembly.[8,9] The association of the LCPs and the peptides is demonstrated to be dependent on the conformation of the peptides but also on the composition of the LCPs with respect to regioregularity, charge and hydrophobic properties. The optical properties of the LCPs were strongly dependent on π-π driven H-aggregation formation that could be modulated by the state of aggregation of the peptide fibers. Surprisingly, the photo luminescence from the LCP was strongly red-shifted when bound to the folded peptides and peptide fibers, which is in contrast to previous observations of LCP-decorated amyloid fibers and LCP/peptide supramolecular assemblies,[5,10] indicating a difference in the mode of interaction depending on the conformation of the scaffold.

Experimental Part

Polypeptide and Polymer Synthesis

The polypeptides JR2E (NAADLEKAIEALEKHLEAKGPVDAAQLEKQLEQAFEAFERAG), JR2K (NAADLKKAIKALKKHLKAKGPVDAAQLKKQLKQAFKAFKRAG), JR2EC and JR2KC were synthesized on a Pioneer automated peptide synthesizer (Applied Biosystems) using standard fluorenylmethoxycarbonyl (Fmoc) chemistry protocols and Fmoc-Gly-poly(ethylene) glycol/polystyrene resin. The crude products were purified by reversed-phase HPLC on a semi-preparative HICHROM C-8 column and identified by MALDI-ToF mass spectrometry. In order to obtain $JR2EC_2$ and $JR2KC_2$, lyophilized peptide monomers (0.001 M) were dissolved in 0.1 M ammonium bicarbonate buffer pH = 8, aerated for 90 min and incubated at 4 °C for at least 24 h before use. Complete oxidation was confirmed using a standard Ellmańs test for determination of free thiols.[11] Details on peptide synthesis are described elsewhere.[7] The synthesis of the LCPs (poly(3,3''-di[thiophene acetic acid)-5,5'' terthiophene) (tPTAA) and poly(thiophene acetic acid) (PTAA) has previously been reported.[5,12] tPTAA has a molecular weight of approximately 1.5 kDa, corresponding to 4–5 monomers whereas PTAA has a broader size distribution and an average molecular weight of 3 kDa, corresponding to about 20 monomers.[5,6]

Optical Measurements

Peptides were stored as lyophilized powders at −20 °C under nitrogen atmosphere until use when stock solutions of 0.001 M in 0.030 M Bis/Tris buffer pH = 7.0 were prepared. The LCPs were dissolved in MilliQ H_2O 18.2 MΩ · cm, PTAA (1 mg · mL^{-1}, corresponding to ca. 360 μM) and tPTAA (1 mg · mL^{-1}, corresponding to ca. 650 μM) and stored at 4 °C protected from light. Sample solutions were prepared at the day of use. For fluorescence experiments, 5 μL of the LCP stock solutions were mixed with 5 μL of polypeptides (10^{-3} M, on a monomer basis) in 65 μL buffer. The solution was further diluted with buffer after 15 min to a final concentration of 6.5×10^{-6} M LCP and 10^{-5} M of polypeptides. Fluorescence emission spectra were recorded using a TECAN Safire^2 plate reader excited at 450 nm using 200 μL wells. Fluorescence imaging was conducted on a Zeiss Axiovert 200M inverted-light microscope equipped with an AxioCam HRc CCD. A mercury lamp with 470/20 nm band pass filter was used as excitation source and 515 nm long pass filter for the emission. The sample solution was incubated on a clean glass slide for a few minutes and the carefully blown away using a nitrogen flow. Circular dichroism spectra were recorded with a CD6 Spectrodichrograph (JobinYvon-Spex) using a 0.1 mm cuvette at room temperature. Each spectrum was collected as an average of three scans in the range 190–260 nm.

Results and Discussion

The 42-residue polypeptides JR2E and JR2K are de novo designed to fold into a helix-loop-helix motif and heterodimerize into four-helix bundles at neutral pH (Figure 1a).[10,13–15] The net-charges of JR2E and JR2K at neutral pH are −5 and +11, respectively, and the charged residues are mainly located at the dimerization interface in order to prevent homodimerization at neutral pH, and the peptide exists as random-coils monomers unless heterodimerized. The driving force for folding is mainly the formation of a hydrophobic core constituted by the hydrophobic inner sides of the amphiphilic helices. The LCP PTAA (Figure 1b) has previously been reported to associate with the polypeptide JR2K and to induce both a helical secondary structure in the peptide and a twisting of the PTAA chain, resulting in a change in its fluorescence spectra and a split-type induced CD (ICD) in the π-π* transition region.[10] The observed ICD pattern was thus not due to formation of π-stacked chiral aggregates, which normally is the case, but rather an effect of induction of main-chain chirality in the LCP because of the interaction between PTAA and the peptide. In the present study, the trimer-based LCP tPTAA (Figure 1b)[5] was not found to induce any ordered secondary structure in neither JR2K nor JR2E, as was determined from their corresponding CD-spectra (Figure 1c). Thus, both peptide monomers appear to adopt a random coil conformation in the presence of tPTAA. Upon heterodimerization and folding of the polypeptides, the CD-spectrum display pronounced minima at 222 and

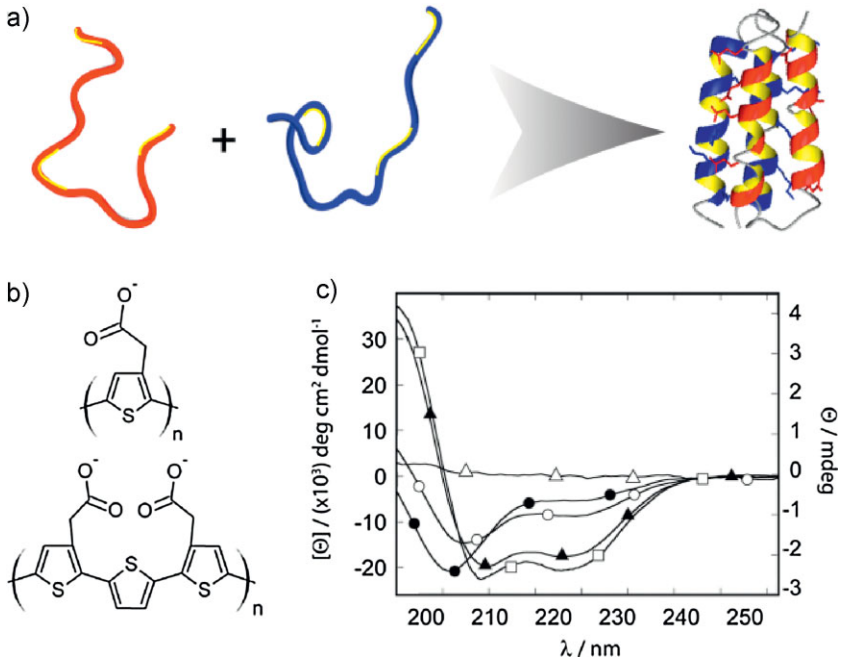

Figure 1. a) The de novo designed helix-loop-helix polypeptides JR2E and JR2K fold into four-helix bundles upon heterodimerization. b) PTAA (top) and tPTAA (bottom). c) Mean residue molar ellipticity of (□) JR2E + JR2K, (○) JR2E + tPTAA, (●) JR2K + tPTAA, (▲) JR2E + JR2K + tPTAA, and ellipticity of tPTAA (△) at pH = 7 in mdeg (right axis).

208 nm with a mean residue molar ellipticity at 222 nm ($[\Theta]_{222\,nm}$) of about $-20\,000 \pm 1000$ deg · cm^2 · dmol^{-1},[13] which is characteristic for helical proteins. When folding in the presence of tPTAA, there was a decrease in the helicity to approximately $-18\,000 \pm 1000$ deg · cm^2 · dmol^{-1}, indicating that the LCP induces a slight perturbation in the polypeptide secondary structure.

The fluorescence emission spectrum of tPTAA is dominated by a peak at 550 nm with a distinct shoulder at around 600 nm (Figure 2b). The ratio of the emission intensity at 550 nm to 600 nm is about 1.25 whereas PTAA demonstrate a spectrum absent of shoulders with a single peak at 590 nm and a 550 to 600 nm ratio of 0.55 (Figure 3a). The tPTAA fluorescence emission spectra were not drastically altered by the presence of any of the two peptides in the random coil state, whereas a significant red-shift of about 50 nm was observed in the presence of the folded heterodimeric four-helix bundle, clearly indicating an interaction between the LCP and the peptide scaffold. A red-shift in the LCP fluorescence spectrum is associated with a planarization of the oligothiophene backbone which increases the effective conjugation length.[16] The planarization typically also increases the possibility for π-stacking and formation of H-type aggregates that result in a quenched emission.[17,18] As quenching was not observed here, the planarization of tPTAA clearly did not result in aggregation of the polymers indicating that the supramolecular complex was dispersed and the polymer chains well separated. The perturbation in the peptide secondary structure, as was seen in the CD-spectrum, further indicate that the oligothiophene backbone to a certain extent is forced into the hydrophobic interior of the four-helix bundle, which presumably is more energetically favorable if the polymer adopts a relatively planar conformation in order to prevent the charged side-groups from entering the hydrophobic core. A planarization of the polymer backbone is further supported by the observation that the association of tPTAA to the four-helix bundles did not result in an ICD pattern in the π-π* transition region (Figure S1, Supporting Information), as was observed for PTAA in the presence of JR2K,[19] which was explained by a twisting of the polymer backbone.

To allow for assembly of the peptides into fibers, the Val in position 22 located in the loop-region, was replaced by a Cys-residue that was further oxidized to form a disulfide-bridge that covalently linked two peptide monomers. The oxidized peptides (referred to as $JR2EC_2$ and $JR2KC_2$) assemble into micrometer long and extremely thin (ca. 5 nm) peptide fibers as a result of a propagating association mediated by folding into four-helix bundles (Figure S2, Supporting Information).[7] Assembly of fibers in the presence of tPTAA not only resulted in a 50 nm red-shift, comparable to the shift seen for the single four-helix bundles, but also a dramatic decrease in the fluorescence emission. The peptide fibers have previously been reported to form fibrous aggregates at low ionic strength,[7] which in this case would bring the LCP chains in closer contact and increase the probability for inter-chain reactions such as non-radiative relaxation. The large red-shift is also in stark contrast to previously reported data of tPTAA and other LCPs when associating to insulin-based amyloid fibers, which produced a blue-shift of the emission peak.[5] Except for a difference in amino acid composition, the two types of fibers comprise two structurally very different motifs. Amyloid fibrils adopt a cross-beta sheet quaternary structure where the β-strands are perpendicular to the fibril axes forming axially aligned extended β-sheets,[20] whereas the polypeptide fibers used here are highly α-helical, further indicating that also the structure of the biomolecular scaffold affects the organization and conformation of the LCP.

Matching vibronic transitions originating from the polymer C = C stretching and ring breathing, which

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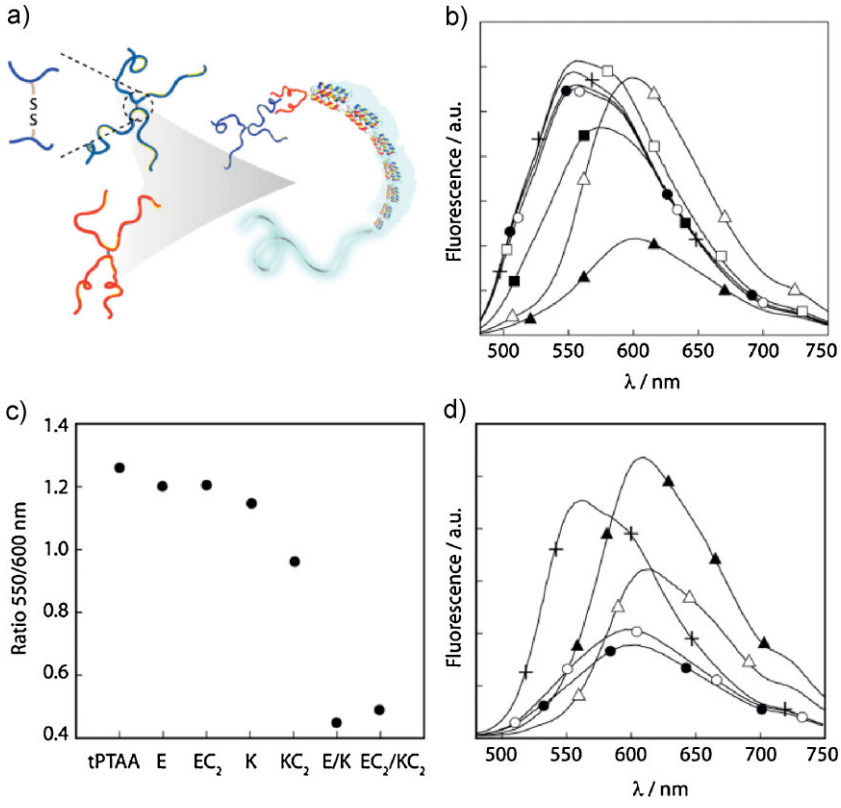

Figure 2. a) The disulfide-linked polypeptides $JR2EC_2$ and $JR2KC_2$ assemble into fibers as a result of folding into four-helix bundles. b) Fluorescence spectra of (+) tPTAA, and tPTAA + (○) JR2E, (□) JR2K, (△) JR2E + JR2K, (●) $JR2EC_2$, (■) $JR2KC_2$, (▲) $JR2EC_2$ + $JR2KC_2$. c) Fluorescence intensity ratio 550/600 nm for tPTAA, and tPTAA in the presence of: (E) JR2E, (EC_2) $JR2EC_2$, (K) JR2K, (KC_2) $JR2KC_2$, (E/K) JR2K + JR2E, (EC_2/KC_2) $JR2EC_2$ + $JR2KC_2$. d) Fluorescence spectra of (+) tPTAA, and (○) tPTAA in 0.050 M NaCl, (●) tPTAA in 0.150 M NaCl, (△) tPTAA and $JR2EC_2$ + $JR2KC_2$ in 0.050 M NaCl (▲) tPTAA and $JR2EC_2$ + $JR2KC_2$ in 0.150 M NaCl.

are typically separated by approximately 0.18 eV in oligothiophenes,[21] to the peaks in the fluorescence spectra of tPTAA when associated to the polypeptide fibers strongly indicate H-aggregate formation. In disordered free H-aggregates, the 0-0 transition is forbidden because of symmetry considerations and the 0-1 transition will hence dominate the spectra.[22,23] The peak at 550 nm (2.25 eV) in the emission spectrum is suggested to correspond to the 0-0 transition, whereas the 0-1 transition is seen at about 600 nm (2.07 eV). The ratio of the 0-0 to 0-1 transitions (Figure 2c) clearly illustrate that the 0-0 transition dominate the spectra of tPTAA in buffer and dispersions containing the monomeric unordered peptides, as expected for single non-bound oligothiophene chains. For dispersions containing the folded polypeptide fiber, the tPTAA 0-1 transition is dominating, in addition to showing a quenched emission as expected from formation of H aggregates. The 0-0 transition is, however, not absent indicating that the H aggregates are not totally disorder free. The random-coil peptide $JR2KC_2$ also induces a red-shift in the tPTAA emission spectrum but the ratio of the 0-0 to 0-1 transitions are close to one, indicating formation of totally disordered aggregates, presumably emerging as a result of charge interactions.

At higher ionic strength, tPTAA and other LPCs tend to aggregate, resulting in a dramatic decrease in emission (Figure 2d). In contrast, the polypeptide fibers are better dispersed at higher ionic strength.[7] When increasing the ionic strength by addition of NaCl to suspensions of the tPTAA-decorated polypeptide fibers, a significant increase in the emission was observed. As the tPTAA emission spectra still remained red-shifted, this strongly indicates that the majority of the tPTAA is bound to the peptide fibers and an increase in the ionic strength separate the aggregated fibers which then lowers the probability of inter-chain reactions between the associated polymers. This is further confirmed by an additional decrease in the 0-0 to 0-1 transition ratio indicating a more well ordered LCP aggregate structure on the dispersed fibers.

The spectral shifts for PTAA, when interacting with the folded polypeptides and polypeptide-fibers, were considerably smaller than for tPTAA and blue-shifted instead of red-shifted (Figure 3a). The disparity in the optical response between the two LCPs is most likely a result of the difference in length and net charge of the polymers. As PTAA has a higher net charge than tPTAA it may to a larger extent prevent fiber aggregation when associating with the peptides. Also the mode of association may differ as tPTAA carry a lower net charge than PTAA, which to a larger extent promotes the association of tPTAA to the folded four-helix bundles through hydrophobic interactions. As compared to PTAA, the polymer backbone of tPTAA can hence be better aligned along the hydrophobic sides of the amphiphilic helices, which explains the observed perturbation in the polypeptide secondary structure and the lack of ICD pattern in the near-UV CD-spectrum of the polymer as this forces tPTAA to adopt a more planar conformation, which was also seen as a red-shifted fluorescence.

In contrast to tPTAA, the association of PTAA to the polypeptide fibers could not be unambiguously confirmed from the fluorescence emission spectra. Notwithstanding the small spectral shift, extensive association of PTAA to the polypeptide fibers was clearly demonstrated using

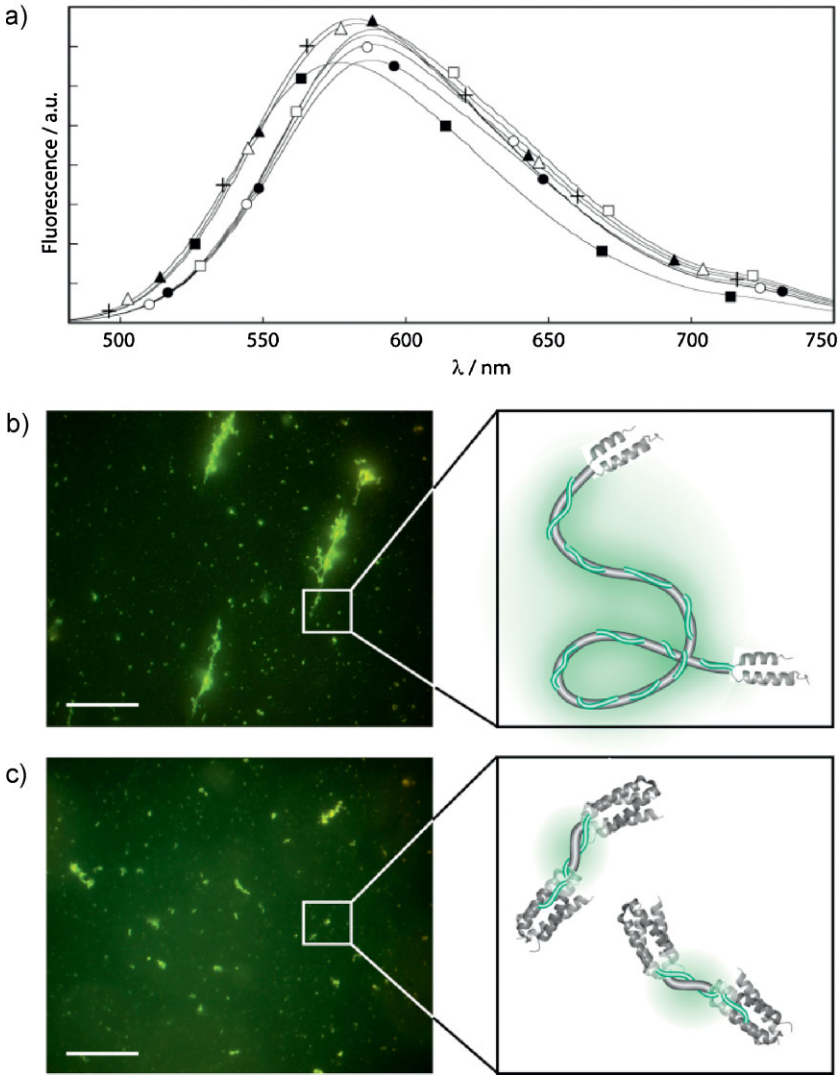

Figure 3. a) Fluorescence spectra of (+) PTAA, and PTAA in the presence of: (○) JR2E, (□) JR2K, (△) JR2E + JR2K, (●) $JR2EC_2$, (■) $JR2KC_2$, (▲) $JR2EC_2 + JR2KC_2$. b) Left: Fluorescence images of stretched and aligned PTAA-decorated polypeptide fibers. Right: Schematic illustration of the proposed arrangement of the LCPs on the polypeptide fibers. c) Left: The size of the fibers was drastically reduced in the presence of JR2E (2.5×10^{-5} M) due to a reduction in size of the fibers as a result of capping of the fiber ends, as schematically illustrated on the right. Scale bars 20 μm.

fluorescence microscopy (Figure 3b). The PTAA/polypeptide fiber assemblies formed large fibrous structures that could be stretched on the substrate through a molecular combing technique using a nitrogen flow prior to imaging.[24] The low background fluorescence indicates that the majority of the LCPs were bound to the fibers. The length of the fibers can be reduced by capping the fiber ends by addition of peptide monomers lacking the Cys residue, which inhibits the fiber growth.[7] The possibility to also control the length of the PTAA-decorated fiber was shown by addition of JR2E, which drastically reduced the size of assemblies (Figure 3c).

Conclusion

In summary, we demonstrate that the LCPs tPTAA and PTAA associate to a set of de novo designed helix-loop-helix polypeptides and polypeptide-based fibers in a folding-dependent manner. Fibers were assembled by hetero-association of disulfide-linked helix-loop-helix and were utilized as a supramolecular template for the LCPs. The association of tPTAA to the folded four-helix bundles and the fibers resulted in a large red-shift in the fluorescence emission spectra due to a planarization of the polymer backbone induced by the assembly of the supramolecular complex. At low ionic strength, the formation of fibrous aggregates enabled polymer inter-chain reactions that resulted in a quenched emission. Albeit forming macroscopic fibrous assemblies with tunable length that could be aligned on a surface, the association of PTAA to the fibers did not result in a large spectral shift, indicating a different mode of assembly as compared to tPTAA. The observed spectral shifts of tPTAA when interacting with the α-helical polypeptide fibers were significantly different from previous studies on amyloid fibers, indicating that the LCPs enable means for discriminating between major secondary structures. The properties of the LCPs as well as the three-dimensional conformation of the polypeptide scaffold thus determine how the polymers are arranged in the supramolecular construct. The ability to control the polypeptide structure and assembly into fibers hence provide a promising route for tuning the optical properties of LCPs and for fabrication of complex supramolecular nanostructures with potential applications in optical devices, biomedical diagnostics and for development of novel synthetic components for organic electronics with well defined structural properties.

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