Covalent Capture of Aligned Self-Assembling Nanofibers

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Supporting Information

ABSTRACT: A great deal of effort has been invested in the design and characterization of systems which spontaneously assemble into nanofibers. These systems are interesting for their fundamental supramolecular chemistry and have also been shown to be promising materials, particularly for biomedical applications. Multidomain peptides are one such assembler, and in previous work we have demonstrated the reversibility of their assembly under mild and easily controlled conditions, along with their utility for time-controlled drug delivery, protein delivery, cell encapsulation, and cell delivery applications. Additionally, their highly compliant criteria for sequence selection allows them to be modified to incorporate protease susceptibility and biological-recognition motifs for cell adhesion and angiogenesis. However, control of their assembly has been limited to the formation of disorganized nanofibers. In this work, we expand our ability to manipulate multidomain-peptide assembly into parallel-aligned fiber bundles. Albeit this alignment is achieved by the shearing forces of syringe delivery, it is also dependent on the amino acid sequence of the multidomain peptide. The incorporation of the amino acid DOPA (3,4-dihydroxyphenylalanine) allows the self-assembled nanofibers to form an anisotropic hydrogel string under modest shear stress. The hydrogel shows remarkable birefringence, and highly aligned nanofibers are visible in scanning electronic microscopy. Furthermore, the covalent linkage induced by DOPA oxidation allows covalent capture of the aligned nanofiber bundles, enhancing their birefringence and structural integrity.

INTRODUCTION

Nature regularly creates highly anisotropic environments, which allows directional movement, aligned application of force, and controlled diffusion. The design of self-assembling systems, particularly “one-dimensional” nanofibers, has advanced considerably in the past decade, and these systems typically self-organize to form a well-structured nanosystem but have little long-range order. In part, this is because of the design of the assembler; it exclusively controls formation of the desired nanofiber but not interfiber interactions. Instead, fiber alignment has relied on more traditional alignment methods, including the use of electric fields, liquid crystals, and mechanical stretching. However, for biocompatible scaffolds, fabrication of macroscopic alignment is still challenging.

Recently, supramolecular chemistry has shown its potential to achieve anisotropic materials. In one case, enzymatic hydrogelation showed that aromatic–aromatic interactions could be used to enhance fibrous alignment. Peptide amphiphiles have also been demonstrated to form aligned monodomain gels via thermally assisted (and other) mechanisms of self-assembly. Strategies for other supramolecular biomaterials are still limited.

Multidomain peptides (MDPs) self-assemble into nanofibers with approximately a 6 nm width and are microns in length. Their self-assembly is based on the ability of hydrophobic packing to stabilize a bilayer structure that is elongated through the formation of a β-sheet hydrogen-bonding network. Their primary sequence can be divided into clearly defined domains: an amphiphilic-core domain composed of alternating hydrophilic–hydrophobic residues and flanking domains comprised of either negatively or positively charged residues. By the addition of salts, the electrostatic repulsion of the charged domains can be quenched, allowing the amphiphilic core to drive nanofiber assembly, and at the appropriate concentration and solvent composition, ultimately generating a three-dimensional hydrogel. In our previous reports, the MDP nanofibers have frequently been observed by electron microscopy and atomic force microscopy to have parallel bundling and short-range alignment. MDPs have also been shown to exhibit shear-thinning and shear recovery. Together, this led us to believe that by tuning the balance of aggregation and fiber growth, MDPs would respond to external shear forces and achieve long-range alignment. As a result of its biological role, as well as its interesting chemical properties, DOPA (3,4-dihydroxyphenylalanine) has been incorporated into synthetic polymers and biomimetic materials. The unique structure of the DOPA side chain allows it to mediate supramolecular interactions, including monodentate and self-bidentate hydrogen-bonding, π stacking, and hydrophobic interactions in aqueous systems.

In addition, DOPA is readily oxidized to α-quinone, followed by a variety of cross-linking reactions. This oxidation-induced cross-linking pathway is orthogonal to the chemistry...
used (simple addition of salts) for self-assembly such that one can be triggered without the other.

In this work, we demonstrate a new self-assembly pathway to form long-range aligned MDP nanofibers with the incorporation of DOPA residues. By tuning both the aromatic feature and hydrophilic substitutions of MDPs, peptide aggregation and fiber formation can be balanced to generate a fibrous scaffold with long-range alignment. Both birefringence and SEM evidence demonstrate the successful formation of the aligned nanostructured strings of MDPs. Additionally, we show that the self-assembled nanostructure can be covalently captured by adding oxidants to trigger DOPA cross-linking. Covalent capture makes the material far more robust under a variety of conditions. This assembly pathway provides a new strategy to design biomimetic materials at the nanoscale with long-range order.

**RESULTS AND DISCUSSION**

**Peptide Design and Characterization.** A series of MDPs with the general sequence of K_{2}(SLXL)_{3}K_{2} were prepared where X was either serine, phenylalanine, tyrosine, or DOPA (herein abbreviated as Z, the structure is shown in Figure 1).

![Figure 1](image1.png)

**Figure 1.** Schematic of multidomain peptides forming aligned self-assembling nanofibers. (A) Chemical structure of DOPA-containing MDP sequence K_{2}(SLZL)_{3}K_{2}. (B) Process of self-assembly, fiber bundling, fiber alignment, and covalent capture. The inclusion of DOPA introduces hydrophobic moieties in addition to the potential for bidentate hydrogen bonding on the surface to reinforce the observed parallel packing of fibers. Upon oxidation, these groups can covalently cross-link with one another or with the amines of the lysine residues.

Our hypothesis is that the inclusion of DOPA would introduce hydrophobic interactions on the surface of nanofibers, whereas the hydroxyl groups would help to maintain the fibrous solubility and provide a second mode of interfiber interaction through bidentate hydrogen bonding. The secondary structure and nanostructure were characterized to confirm the peptide self-assembly (Figure 2). FTIR exhibited an amide I parallel peak near 1630 cm^{-1} and antiparallel: 1695 cm^{-1}, which together can be correlated to an antiparallel β-sheet structure. The CD spectrum containing a maximum at 195 nm and a minimum at 216 nm for β-sheets. (C) Negative-stain TEM and (D) SEM both revealed similar fibrillar structure to the unmodified K_{2}(SL)L_{3}K_{2} peptide. Scale bar for C is 50 nm. Scale bar for D is 100 nm.

![Figure 2](image2.png)

**Figure 2.** Structural characterization of K_{2}(SLZL)_{3}K_{2}. (A) FTIR spectrum showing characteristic peaks for β-sheet: 1630 cm^{-1} and antiparallel: 1695 cm^{-1}. (B) CD spectrum showing characteristic maximums at 195 nm and minimums at 216 nm for β-sheets. (C) Negative-stain TEM and (D) SEM both revealed similar fibrillar structure to the unmodified K_{2}(SL)L_{3}K_{2} peptide. Scale bar for C is 50 nm. Scale bar for D is 100 nm.

**Long-Range Shear-Induced Alignment.** The above characterization was performed on nanofibrous MDP hydrogels which had been prepared by simple mixing of the MDP with HBSS buffer. To examine the possibility of shear alignment of these nanofibers, K_{2}(SLZL)_{3}K_{2} was prepared in a gel-loading tip and slowly injected into the HBSS buffer solution while dragging the pipet backward. Gelation occurs immediately upon mixing and resulted in the formation of a narrow string of hydrogel which was inches long (Figure 3A,B). The peptide string showed a unique mechanical strength in that it can be easily lifted by tweezers without breaking. Examination of the string by polarized optical microscopy revealed strong birefringence along the length of the fiber (Figure 3C). This suggests that the self-assembled MDP formed an inches-long anisotropic string.

To observe the organization of the nanofibers inside the string, SEM was performed. As showed in Figure 3D, the string was found to be composed of aligned bundles of nanofibers, completely different from what was observed in our simple mixing experiment. Consistent with the birefringence results, this nanofiber alignment was also found extending along the peptide string. Figure S3 also showed the edge section of the string, which revealed that the alignment also exists in the interior of the fiber. The string was broken by pipet mixing and sonication for TEM sample deposition. Aligned nanofiber bundles were frequently observed on the TEM grids (Figure S4). Together, these results show that with careful injection of...
the MDP into the appropriate buffer solutions creates long strings of aligned MDP nanofiber bundles.

In order to evaluate the role of DOPA, K₂(SL)₃K₂ peptide strings were also made in the same manner. However, K₂(SL)₃K₂ strings displayed no birefringence (Figure S2, C and D), which suggested that there is no internal alignment. Additionally, the formed string had poor mechanical strength and easily fractured. Considering the structural difference between K₂(SL)₃K₂ and K₂(SLZL)₃K₂, the aromatic side chain of DOPA may be playing a key role in the difference in long-range organization. Two additional MDPs were prepared to help elucidate this difference in alignment.

K₂(SLFL)₃K₂ and K₂(SLYL)₃K₂. In the investigation of long-range alignment formation, K₂(SLFL)₃K₂ and K₂(SLYL)₃K₂ were synthesized. This allows us to examine MDPs with only small differences in side chain chemistry: phenylalanine with a benzyl ring but no hydroxylation, tyrosine with one hydroxyl group, and DOPA with two. The peptides were dissolved in water to make 2 wt % solutions. When we injected the MDP solutions into HBSS buffer as described above, strings were formed in both cases but did not show birefringence, which was similar to K₂(SL)₆K₂ (Figure S6). Also, these peptide’s strings were not strong enough to be lifted from solution or otherwise manipulated after creation. Instead, the hydrogel strings easily fragmented.

The peptides were characterized by FTIR, CD, TEM, and rheology. In FTIR and CD characterizations, they showed similar spectra to those of K₂(SLZL)₃K₂ (Figure 4A,B). The results revealed that both peptides containing aromatic substitutions still self-assemble into an antiparallel β-sheet structure. However, when we added HBSS buffer to the phenylalanine- or tyrosine-containing MDP solutions, both became cloudy and started precipitating. This was unlike K₂(SLZL)₃K₂, which formed a clear hydrogel. Examination of the MDP suspension by TEM revealed that their nanostructures have a morphology different from K₂(SLZL)₃K₂. As shown in Figure 4C, K₂(SLFL)₃K₂ formed fibers which appear shorter and more rigid. Additionally, we frequently observe this MDP forming small bundles composed of two to four fibers running in parallel. K₂(SLYL)₃K₂ assembled into longer, more flexible-looking nanofibers in which parallel bundles are not as frequently observed. Additionally, these nanofibers displayed increased physical entanglement as compared to K₂(SLFL)₃K₂ (Figure 4D). In rheological characterization (Figure S5, C and D), K₂(SLFL)₃K₂ showed a poor G’ value of 45 Pa while K₂(SLYL)₃K₂ had a high G’ value of 537 Pa. This supports our qualitative TEM observation that K₂(SLYL)₃K₂ had more physical entanglement.

Contrastively, K₂(SLZL)₃K₂ forms much longer nanofibers because, in part, of the improved solvation of the increased hydroxylation of the DOPA side chain. In cast hydrogels, these fibers are kinetically trapped by physical entanglement and are rarely observed to bundle (Figure 2C). However, upon extrusion into the HBSS buffer through a narrow bore needle, fibers are forced into parallel alignment. Once aligned, this arrangement persists, potentially stabilized through the unique bidentate hydrogen bonding known to occur between DOPA residues. 21

DOPA Oxidation and Covalent Capture. To evaluate the effect of oxidation on long-range alignment, we used sodium periodate, a commonly used chemical oxidant for DOPA. 26,27 Ortho-quinone, the periodate-oxidized product of DOPA, has been reported to cross-link with a variety of amino acids, such as lysine, histidine, and cysteine. 25 The UV-vis spectra of K₂(SLFL)₃K₂ treated with sodium periodate were monitored over 24 h (Figure 6A). Before the addition of sodium periodate, the solution only displayed a λmax at 280 nm, which represents the absorption of DOPA. 27 After 10 min of periodate addition, two shoulder peaks at 305 and 400 nm appeared. The two peaks match the expected λmax values of DOPA-quinone, revealing the quick oxidation of DOPA. After 24 h, the spectrum shows two broad peaks from 270 to 300 nm and from 490 to 520 nm, which can be assigned to a mixture of the Michael addition product with lysine and di-DOPA products. 25,50 DOPA oxidation was also assessed by the rheological
orthoa-quinone appeared ($\delta$ 6.19, 6.39, 7.09) within 10 min. After 1 h, DOPA peaks were not observable, suggesting DOPA was fully converted to ortho-quinone. After 24 h, all quinone peaks disappeared because of the loss of aromatic hydrogens during cross-linking and decreased isotropic tumbling. In agreement with the UV−vis analysis, the NMR result suggests that periodate triggers DOPA oxidation in less than 10 min and the conversion to cross-linked structures is completed during the following 24 h. MALDI-TOF spectra (Figure 5C) of the oxidized K$_2$(SLZL)K$_2$ shows peaks for the monomer through the tetramer and possibly higher oligomers. SDS−PAGE (Figure S8) also suggests a high molecular weight, covalently cross-linked system. Collectively, this evidence indicates that the K$_2$(SLZL)K$_2$ peptide can be oxidized in the presence of periodate and ultimately forms via covalent capture through DOPA cross-linking.

K$_2$(SLZL)K$_2$ strings were formed in HBSS as before, but this time the HBSS contained 29.3 mM sodium periodate (1 equiv to peptide). Whereas K$_2$(SLZL)K$_2$ self-assembled into aligned nanofiber bundles, DOPA groups are oxidized to DOPA-quinone followed by covalent cross-linking. After 24 h, the strings were examined by polarized microscopy and found to have uniform birefringence (Figure 6C−E). The red color of the strings is related to DOPA-quinone formation and subsequent cross-linking. In the SEM (Figure 6B), it was found that the oxidized DOPA nanofibers formed straight and highly aligned arrays, consistent with the uniform birefringence of the strings.

To analyze the K$_2$(SLZL)K$_2$ string formation process without oxidation, dithiothreitol (DTT) was used to provide a reducing environment. Without DOPA oxidation, the peptide strings still showed birefringence, but it was weaker than it was for the oxidized strings (Figure 7A−C). The SEM also showed that the nanofibers were aligned locally but the bundles were flexible, allowing bends and turns (Figure 7D,E).

Figure 5. Oxidative cross-linking of K$_2$(SLZL)K$_2$. (A) Scheme showing oxidation of DOPA and possible resulting covalent cross-links. (B) $^1$H NMR, DOPA, and ortho-quinone: the protons in the aromatic region ($\delta$ 6.58, 6.68, 6.75) indicated that the sample contained only nonoxidized DOPA peptide. Upon the addition of 1 equiv of periodate, ortho-quinone appeared ($\delta$ 6.19, 6.39, 7.09) within 10 min. After 1 h, DOPA peaks were not observable, suggesting DOPA was fully converted to ortho-quinone. After 24 h, all quinone peaks disappeared because of the loss of aromatic hydrogens during cross-linking and decreased isotropic tumbling. In agreement with the UV−vis analysis, the NMR result suggests that periodate triggers DOPA oxidation in less than 10 min and the conversion to cross-linked structures is completed during the following 24 h. MALDI-TOF spectra (Figure 5C) of the oxidized K$_2$(SLZL)K$_2$ shows peaks for the monomer through the tetramer and possibly higher oligomers. SDS−PAGE (Figure S8) also suggests a high molecular weight, covalently cross-linked system. Collectively, this evidence indicates that the K$_2$(SLZL)K$_2$ peptide can be oxidized in the presence of periodate and ultimately forms via covalent capture through DOPA cross-linking.

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Figure 7. K2(SLZL)3K2 forming strings with weak birefringence and wavy bundles with the presence of dithiothreitol (DTT). (A)−(C) Birefringence and (D), (E) SEM evidence suggesting discontinuous orientation of the nanofiber alignment. Scale bars for A−C are 500 μm. Scare bars for D and E are 1 μm.

As a result, the relatively poor orientation of the nanofiber alignment weakened the birefringence of the strings. We moved both the periodate-treated and DTT-treated strings into deionized water, which removed the phosphate ions required for the ion-bridging that triggers self-assembly. The shape of the strings and their birefringence was monitored to evaluate the integrity of long-range alignment. Before the strings were moved to water, both oxidized strings (Figure 8A) and nonoxidized strings (Figure 8C) were straight and showed birefringence. After 24 h, the shape and birefringence of the oxidized peptide strings showed no change (Figure 8B), indicating the integrity of the long-range alignment. Without the help of phosphate ions, the covalent cross-links induced by oxidation still bundled the aligned nanofibers, leaving the strings and birefringence unchanged. In contrast, the nonoxidized strings swelled and became fragmented in less than 10 min after being moved to water (Figure 8D). Similarly, the string fragments lost their birefringence, demonstrating that without either ionic or covalent cross-linking the MDP nanofibers and their bundling began to disassemble.

Utilization of DOPA residues in MDPs provides a watersoluble hydrophilic domain to allow self-assembly into nanofibers, yet also contains hydrophobic packing and bidentate hydrogen-bonding characteristics sufficient for stabilizing interfiber interactions that allow these nanofibers to align into parallel bundles upon shearing. DOPA can then be easily oxidized to initiate covalent cross-linking within and between fibers. These covalently captured strings are mechanically strong enough to manipulate and chemically resilient enough to survive transfer into media which, without the covalent cross-links, would result in peptide disassembly. The characteristics of the K2(SLZL)3K2 nanofiber strings make them attractive materials for a variety of biomaterial applications.

### EXPERIMENTAL METHODS

All chemicals not otherwise specified were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

**Peptide Synthesis.** K2(SLZL)3K2, K2(SLYL)3K2, K2(SLFL)3K2, and K2(SLYL)3K2 were synthesized using solid-phase peptide synthesis methodology using a protocol previously reported by us. All resin and coupling reagents were purchased from EMD Chemicals (Philadelphia, PA). After cleavage from the resin, TFA was removed by rotoevaporation. The crude peptides were dialyzed for 3 days using 1000 Da MWCO dialysis tubing (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, CA) against Milli-Q deionized water. K2(SLZL)3K2 was dialyzed at pH 3 to help avoid oxidation. The dialyzed peptide solutions were frozen, lyophilized, and then stored at −20 °C. All peptides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, using Autoflex MALDI-TOF MS (Bruker Instruments, Billerica, MA), to verify the correct mass (see Supporting Information).

**Circular Dichroism (CD).** For CD assessment, the peptide solutions were diluted to 0.01 wt % with water and adjusted to pH 7. The spectra were recorded from 190 to 250 nm with a 0.1 nm data pitch using a Jasco-810 spectropolarimeter (Jasco Inc., Easton, MD). The scan speed was 50 nm/min and the signal was averaged over 5 scans.

**Fourier Transform Infrared Spectroscopy (FTIR).** Aqueous peptide solutions (0.1 wt %) were adjusted to pH 7 and allowed to dry on the diamond of a “Golden Gate” for attenuated total reflectance (ATR) measurement. The spectra were collected and accumulated from 64 scans on a Jasco FT/IR 660 plus spectrometer (Jasco Inc., Easton, MD).

**Transmission Electron Microscopy (TEM).** For TEM samples, serial dilutions were performed with Milli-Q water to reach a peptide concentration of 0.01 wt %. The diluted sample solutions were pipetted onto a Quantifoil R1.2/1.3 holey carbon-mesh copper grid and allowed to sit for 1 min. Excess solution was wicked away with filter paper, and the grid was negatively stained with 2 wt % pH 7 phosphotungstic acid (PTA) for 5 min, followed by being dried overnight. Imaging was performed at 100 kV on a JEOL 2010 transmission electron microscope (JEOL USA Inc., Peabody, MA).

**Hydrogel Formation.** To prepare MDP hydrogels, lyophilized peptide solutions were dissolved to 2 wt % in Milli-Q water, adjusted to pH 7, and subsequently diluted 50:50 with 1× Hank’s Buffered Salt Solution (HBSS; Life Technologies), which contains 1.26 mM CaCl2, 5.33 mM KCl, 0.44 mM KH2PO4, 0.5 mM MgCl2·6H2O, 0.41 mM MgSO4·7H2O, 138 mM NaCl, 4 mM NaHCO3, 0.3 mM Na2HPO4, and 5.6 mM glucose. To prepare the reduced DOPA hydrogel, 2 wt % peptide solution was dialyzed 50:50 with HBSS containing 293 mM dithiothreitol (DTT) (10 eq. to DOPA concentration). To prepare the oxidized DOPA hydrogel, 2 wt % peptide solution was dialyzed

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50:50 with HBSS containing 29.3 mM NaIO₄ (1 eq. to DOPA concentration). The solution was further diluted to 0.02 wt % for UV–vis absorption measurement using a TECAN Infinite 200 plate reader (Tecan US, Inc., Morrisville, NC). For MS analysis, 1 eq. NaIO₄ was added to prepare the oxidized DOPA hydrogel. After 24 h of oxidation, the hydrogel was dialyzed against Milli-Q deionized water for salt removal. The gel was then homogenized by sonication and further diluted to 0.05 wt %.

**Peptide-String Formation.** To form peptide strings, the peptides were dissolved to 2 wt % in Milli-Q water. MDP strings were prepared by injecting the 2 wt % peptide solution into HBSS buffer from a Fisherbrand Gel-Loading Tip, 1–200 μL (Thermo Fisher Scientific, Waltham, MA). The tip was dragged backward simultaneously with the injection rate. For the oxidized peptide string, 2 wt % K₂(SLZL),K₂ aqueous solution was injected into HBSS containing 29.3 mM NaIO₄. The peptide string was stored in the oxidizing medium for 24 h to allow the DOPA oxidation to complete. The medium was then replaced by Milli-Q water. For reduced peptide string, 2 wt % K₂(SLZL),K₂ aqueous solution was injected into HBSS containing 293 mM DTT. The shape and birefringence of the string were subsequently monitored to evaluate the integrity of alignment.

**Rheological Analysis.** The hydrogels were prepared 24 h before measurement as the protocol described in Hydrogel Formation. The storage modulus (G″) and loss modulus (G′″) were monitored using a TA Instruments AR-G2 rheometer (TA Instruments, New Castle, DE). Prepared hydrogel was deposited (150 μL) onto the rheometer stage and a 12 mm stainless-steel parallel plate was used with a 1000 μm gap height. In the strain sweep analysis, G″ and G′″ were monitored under an applied strain of 0.01% to 200% at a frequency of 1 rad/s. In the frequency sweep analysis, G″ and G′″ were monitored under 1% strain at a frequency of 0.1 rad/s to 100 rad/s. Shear recovery experiments were performed by subjecting the gel to 1% strain for 20 min, increasing the strain to 200% for 1 min, and then reducing the strain back to 1% for 20 min.

**Nuclear Magnetic Resonance Spectroscopy (NMR).** All peptides were dissolved in D₂O to 0.2 wt % for measurement. The spectra were collected and accumulated from 16 scans on a Bruker AVANCE III HD 600 MHz High Performance Digital NMR (Bruker Instruments, Billerica, MA).

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**REFERENCES**


**SUPPORTING INFORMATION**

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b04655.

MALDI-TOF mass spectra, rheological data, TEM and SEM images, SDS-PAGE gel image (PDF)