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Metal-Triggered Collagen Peptide Disk Formation

David E. Przybyla and Jean Chmielewski*

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907

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Collagen plays a ubiquitous role as a biological scaffold in a range of biological settings, such as bone, cartilage, and the extracellular matrix. As such, natural collagen has been evaluated for numerous uses in regenerative medicine and tissue engineering.[1] The unique biological and structural properties of these materials can be traced back to repeating Xaa-Yaa-Gly sequences within collagen, wherein Xaa and Yaa are predominately proline and hydroxyproline, respectively. These repeating sequences lead to a type-II polyproline (PPII) helix within each collagen strand, and these helices associate to form a collagen right-handed triple-helical structure.

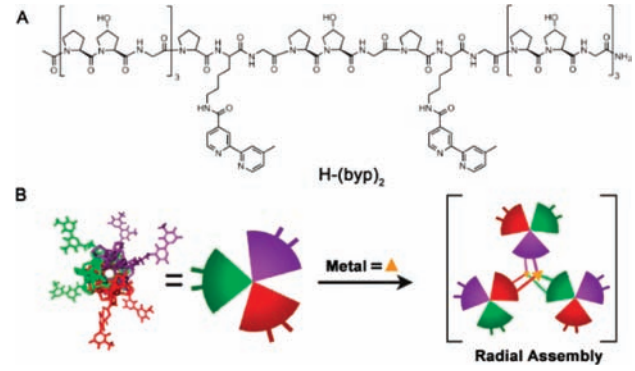
The ability to synthetically modify collagen peptides has resulted in a greatly increased understanding of the stability of the triple helix.[2] More recently, there has been interest in manipulating the structure of collagen peptides to facilitate higher-order organization, often accomplished through self-assembly strategies. These hierarchical approaches have used specific structural cues or sticky ends to promote programmable assembly. For instance, collagen peptide-based fibrils and fibers,[3] meshes,[4] and microflorettes[5] have been generated, and some of these materials have been harnessed for specific applications, such as blood platelet activation,[3d,h] nanowire formation,[6] and three-dimensional cell culture.[4]

To expand the number of strategies for assembling collagen peptide materials, we probed how a radial assembly mechanism could control the morphology of a metal-triggered collagen peptide. We envisioned that the use of two staggered bipyridine ligands along a collagen peptide would provide multiple sites for metal coordination within a triple helix and potentially add rigidity to metal-promoted radial assemblies. To achieve this, **H-(byp)$_2$** (Scheme 1A) was designed with repeating Pro-Hyp-Gly sequences and with bipyridine ligands conjugated to lysines at the fourth and sixth trimer positions. Subsequent triple helix formation would result in the staggering of six bipyridine ligands along the triple helix, which would then be capable of assembling radially upon addition of metal ions (Scheme 1B).

H-(byp)$_2$ was synthesized by solid-phase methods using established procedures.[3f] The resulting material was purified to homogeneity by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry. Circular dichroism (CD) studies confirmed that **H-(byp)$_2$** exhibited a PPII helix, with a maximum at 225 nm both in the absence and presence of Fe(II)$(ClO_4)_2$ (see the Supporting Information).[3] Thermal denaturation studies confirmed that a stable triple helix could form with **H-(byp)$_2$**, even with the addition of the two bipyridine moieties (see the Supporting Information), although a 30 °C drop in thermal stability relative to $(POG)_9$ was observed. The addition of Fe(II), however, was found to increase the melting temperature of **H-(byp)$_2$** from 39 to 52 °C, a trend that is consistent with other designed metal-binding collagen peptides.[7,3f]

UV−vis spectroscopy was used to monitor metal ion binding with **H-(byp)$_2$** and to measure the binding stoichiometry (Figure 1A). Addition of Fe(II) to a solution of **H-(byp)$_2$** resulted in an

Scheme 1. Self-Assembling Collagen Peptide: (A) Peptide Sequence of **H-(byp)$_2$**; (B) Triple Helix Formed from **H-(byp)$_2$** with Six Bipyridine Ligands Mediating Metal-Promoted Radial Assembly



observed maximum absorbance of 540 nm, a value consistent with the known metal-to-ligand charge transfer transition of a bipyridine/Fe(II) complex (see the Supporting Information).[8] Also, the addition of increasing Fe(II) concentrations to a **H-(byp)$_2$** solution resulted in an observed Fe(II)/**H-(byp)$_2$** molar ratio of 2:3, consistent with the expected bidentate coordination of bipyridine with Fe(II). Furthermore, the reversibility of the assembly process was confirmed by the addition of the metal chelator EDTA (100 μM) and the ensuing loss of the absorbance at 540 nm (Figure 1A).

The assembly of **H-(byp)$_2$** was monitored by dynamic light scattering (DLS) (Figure 1B). **H-(byp)$_2$** (1 mM) in the absence of Fe(II) displayed two distinct size distributions, a phenomenon that has been observed with other collagen peptide-assembling systems.[3d] The smaller assembly had a hydrodynamic radius of 3 nm, which is consistent with those of other monomeric triple-helical peptides.[3d,f] The existence of a larger assembly (75 nm radius) indicated that multiple **H-(byp)$_2$** peptides could aggregate even in the absence of metal ions, potentially through interactions between the bipyridine ligands on adjacent triple helices. Upon the addition of Fe(II) (1 mM) to **H-(byp)$_2$** (1 mM), the formation of an even larger assembly (300 nm radius) was observed, with the complete loss of the particle size attributed to the monomeric triple helix. These data indicate that Fe(II) facilitates higher-order assembly, presumably through the coordination of bipyridine ligands on multiple triple helices. The reversibility of the metal-promoted assembly was confirmed by DLS, as the addition of EDTA (10 mM) to the above solution resulted in particle sizes that were similar to the premetal **H-(byp)$_2$** sample.

Transmission electron microscopy (TEM) imaging revealed that **H-(byp)$_2$** formed ill-defined aggregates in the absence of metal (see the Supporting Information). However, upon the addition of Fe(II), **H-(byp)$_2$** was found to assemble into a round, disklike morphology with diameters ranging from 50 to 500 nm (Figure 2A). The morphology of these disklike structures was further probed by atomic force microscopy (AFM) (Figure 2C). AFM analysis confirmed that the round structures observed by TEM were indeed

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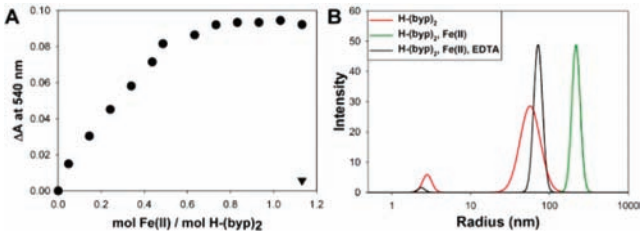

Figure 1. (A) UV−vis titration of **H-(byp)$_2$** (20 μM) with Fe(II) (●) and after addition of EDTA (100 μM) (▼). (B) DLS data for **H-(byp)$_2$** (1 mM) alone (red) and after addition of Fe(II) (1 mM) (green) and then EDTA (10 mM) (black) in 10 mM HEPES buffer (pH 7.0).

disks with a height of ∼10 nm (Figure 2C). The height of the collagen peptide disks was similar to the length of the triple helix of **H-(byp)$_2$** (∼10 nm), raising the possibility that the observed disks were formed through a radial growth mechanism as outlined in Scheme 1B. It is interesting to compare the disk structures obtained with **H-(byp)$_2$** (Figure 2A) with the branched fibers that were obtained from **H-byp**, in which a single bipyridine ligand was incorporated into the collagen peptide (Figure 2B).[3f] We hypothesized that flexibility between the ligand and the collagen triple helix in **H-byp** may have resulted in overhangs that facilitated linear growth into fibers. By increasing the rigidity between the metal-chelated triple helices with an additional bipyridine unit per peptide, radial expansion was promoted, leading to a completely different higher-order structure.

We probed whether we could alter the size or shape of the metal-promoted collagen peptide assemblies by changing the Fe(II)/**H-(byp)$_2$** ratio. For instance, we held the **H-(byp)$_2$** concentration constant at 500 μM and scanned a range of Fe(II) concentrations from 250 to 1000 μM. In all cases, TEM analysis demonstrated the same morphology of the assemblies as described above: round and disklike with diameters ranging from 100 to 500 nm (see the Supporting Information). Similarly, altering the overall concentration of equimolar mixtures of Fe(II) and **H-(byp)$_2$** did not alter the relative size or shape of the assemblies (see the Supporting Information). Additional metal ions were also screened to probe the metal dependence of collagen peptide assembly. Specifically, when **H-(byp)$_2$** (1 mM) was incubated with Fe(II), Co(II), or Zn(II) (1 mM), round, disk-like assemblies were observed by TEM, whereas incubation with Cu(II) led to the same ill-defined aggregates as observed with **H-(byp)$_2$** alone (see the Supporting Information). These data suggest that the metal-promoted radial assembly of **H-(byp)$_2$** into a disk morphology may be dependent upon octahedral coordination of the metal ion with the bipyridine ligands along the triple helix.

In summary, we have shown that designed radial assembly of collagen peptide triple helices can be realized upon the addition of metal ions. This hierarchical assembly resulted in collagen peptide disks, a structure that has not been observed previously in other higher-order collagen peptide assemblies. Furthermore, the assembly occurred in the presence of a variety of metal ions and was reversible upon the addition of EDTA. The nanopatterning of materials has previously been demonstrated for a range of applications, including cell adhesion and stem cell differentiation.[9] Also, natural collagen disks have been fabricated for a number of uses, including corneal drug delivery and bone formation.[10] We therefore envision that by combining collagen's ubiquitous natural functions with programmable assembly strategies, unique collagen surfaces with customizable biological functions may be generated.

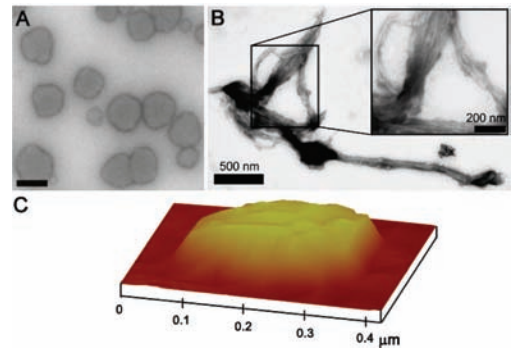

Figure 2. (A) TEM image of **H-(byp)$_2$** (1 mM) with Fe(II) (1 mM). The scale bar represents 500 nm. (B) TEM image of **H-(byp)** (1 mM) with Fe(II) (1 mM).[3f] (C) AFM image of **H-(byp)$_2$** (1 mM) with Fe(II) (1 mM). The experiments were carried out in 10 mM HEPES buffer (pH 7.0).

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Supporting Information Available: Additional experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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