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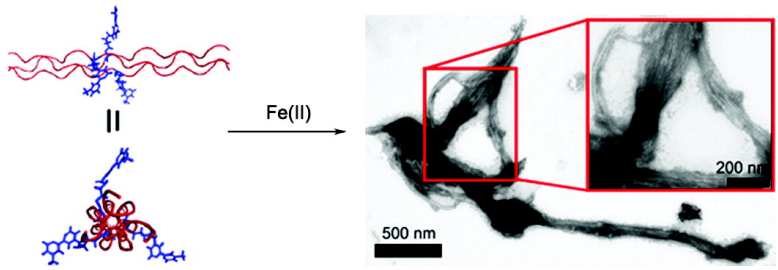

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Metal-Triggered Radial Self-Assembly of Collagen Peptide Fibers

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Collagen is one of the major components of the extracellular matrix and encompasses skin, bone, tendons, ligaments, and blood vessels. Collagen fibers are composed of bundled triple helices consisting of three repeating Xaa-Yaa-Gly strands, where Pro-HypGly (Hyp = (2*S*,4*R*)-4-hydroxyproline) is the most frequently observed unit. Currently collagen has multiple applications in cell adhesion, tissue regeneration, and drug delivery.[1] As a result, there is an interest in creating synthetic collagen fibers that can be used not only to mimic native collagen but also to enhance its biological roles.[2]

One such approach has been to generate small collagen peptides that self-assemble into collagen fibers. To date, the reported strategies for generating these self-assembling collagen fibers have employed linear growth through incorporation of a variety of N- and C-terminal sticky ends. Specifically, electrostatic interactions,[3] $\pi-\pi$ stacking,[4] a modified cysteine knot,[5] and native chemical ligation[6] have been implemented. While these strategies have been successful in generating collagen peptide fibers, there is still a need to control the three-dimensional architecture of collagen networks, a major necessity for tissue engineering. With this in mind, we sought a means for incorporating an additional dimension of growth into collagen peptide fibers.

In this report, we describe a design for metal-triggered radial growth of a collagen triple-helix into collagen fibers (Figure 1). The metal trigger was implemented because of its versatility in other polypeptide systems and known ability to control the structure in metal−organic structures.[7] The design relies on a repeating sequence of POG with a bipyridyl-modified lysine residue in place of the hydroxyproline residue in the central tripeptide (Figure 1a, **H-byp**). We hypothesized that this single amino acid modification would still allow for stable collagen triple helix formation on the basis of numerous host−guest collagen peptide studies.[8] The position of the three bipyridyl ligands in the center of the triple helix yields three potential directions for radial growth (Figure 1b). Upon the addition of metal ions, multiple triple helices can self-assemble in a radial direction, potentially yielding three-dimensional collagen networks (Figure 1c).

The peptide **H-byp** was synthesized by standard solid-phase synthesis on a ChemMatrix rink amide resin via HBTU coupling using Lys(Mtt)-OH in the central position. The removal of the Mtt protecting group was performed on the solid support with DCM/TFA (98:2), and the free amine was subsequently coupled with 4′-methyl-2,2′-bipyridine-4-carboxylic acid. The peptide was cleaved from the resin with TFA/TIPS/H_2O (95:2.5:2.5), purified to homogeneity by reverse phase HPLC, and characterized by MALDI-TOF mass spectroscopy.

The circular dichroism (CD) spectrum of **H-byp** was examined to determine if the peptide formed a stable triple helix and to investigate the effect of added metal ions. The CD spectrum of **H-byp** (250 μM) displayed a typical collagen triple helix profile with a maximum at 225 nm, and addition of metal ion, such as Fe(II), had no effect on the CD spectrum, confirming that a triple helix also formed under these conditions (see Supporting Information). Thermal denaturation studies were performed with **H-byp** to determine the stability of its triple helix. Although somewhat less stable then $(POG)_9$ (T_m of 67 °C, data not shown), a T_m of 56 °C was observed for **H-byp** (see Supporting Information). However, in the presence of the metal ion Fe(II), the T_m increased to 63 °C. This increase in thermal stability with added metal ion could be attributed to metal-promoted assembly of multiple triple helixes or to intrastrand coordination within a single triple helix. Furthermore, upon the addition of EDTA (100 mM) the T_m returned to 57 °C, indicating that the enhanced metal-promoted stability was also reversible.

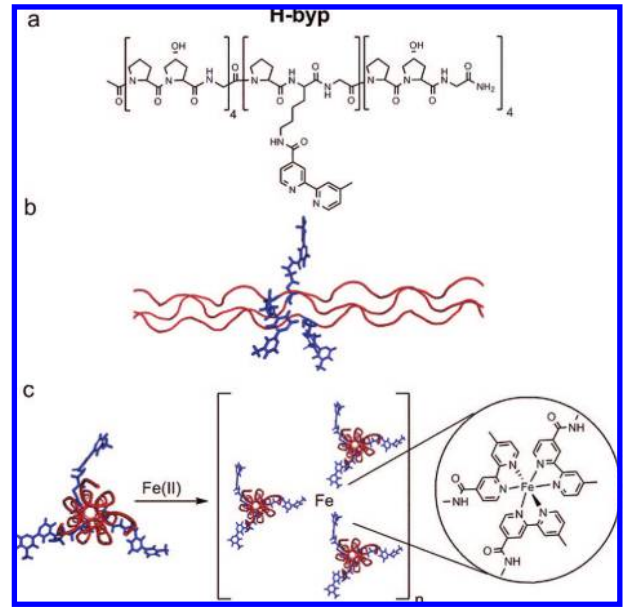

Figure 1. Collagen-mimetic peptide, triple helix, and metal-triggered assembly: (a) amino acid sequence of **H-byp**; (b) side view of **H-byp** after triple helix formation (peptide, red; bipyridine modification, blue); (c) top view of a single triple helix followed by metal-triggered assembly.

UV−vis titration was used to confirm the presence of a complex between **H-byp** and Fe(II), and determine the binding stoichiometry (Figure 2a). It has previously been established that the metal to ligand charge transfer resulting from the binding of bipyridine with Fe(II) generates an absorbance maximum of 540 nm.[9] The addition of Fe(II) to a **H-byp** solution (54 μM) generated a magenta solution with a maximum absorbance at 540 nm. A maximum absorbance was observed at a relative molar ratio of 1:3 Fe(II):**H-byp**, consistent with the bidentate coordination of bipyridine to octahedral Fe(II) (Figure 1c).

Dynamic light scattering (DLS) was used to determine if the addition of Fe(II) created larger ordered species with the collagen peptide (Figure 2b). A solution of **H-byp** (1 mM) was preheated to 70 °C followed by addition of Fe(II) (0.5 mM) and 4 day

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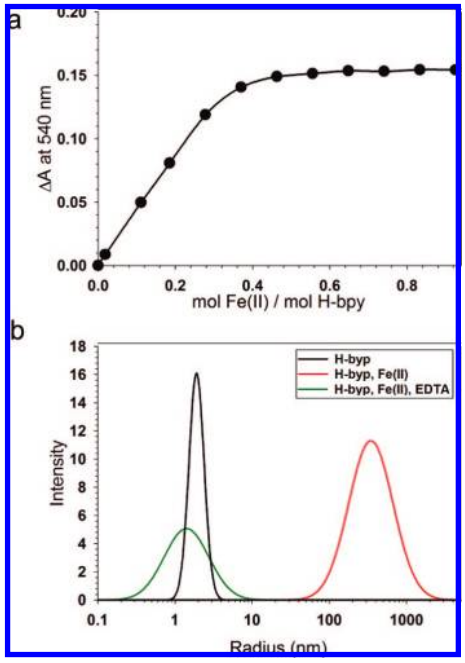

Figure 2. (a) UV−vis titration of **H-byp** (54 μM) with Fe(II); (b) dynamic light scattering of **H-byp** (1 mM), in 10 mM HEPES pH 7.0 (black), with Fe(II) (500 μM) (red), and with Fe(II) (500 μM) and EDTA (100 mM) (green).

incubation at 20 °C. A hydrodynamic radius of approximately 3 nm was observed for **H-byp**, consistent with other collagen triple helical peptides.[4] However, in the presence of Fe(II) a broad distribution of radii were observed with a mean radius of 500 nm. This suggested that the presence of Fe(II) facilitated the assembly of larger aggregates. At lower peptide concentrations (250 and 50 μM) larger assemblies were also observed (mean radii of 200 and 150 nm, respectively) in conjuction with monomeric triple helices (see Supporting Information). Next, we sought to investigate the reversibility of the assembly by adding the metal chelator EDTA. The data indicated that the assembly was completely reversible and the assembled peptide returned to its monomeric triple helix. A screen of other metal ions, such as Cu(II), Zn(II), and Ni(II) (see Supporting Information) was also performed to determine if the assembly process was specific to different metal ions. Of these, Cu(II) was also found to promote the assembly of **H-bpy**.

Transmission electron microscopy (TEM) was used to visualize the morphology of the assembled **H-byp**−Fe(II) species. A solution containing **H-byp** (2 mM) was preheated to 70 °C followed by the addition of Fe(II) (0.3 mM) and a 4 day incubation at 4 °C (Figure 3). These conditions were implemented because previous studies have found that preheating collagen like peptides to temperatures near their T_m promote fibrillogenesis.[10] Fibers were consistently observed with lengths on the order of 3−5 μm, and a number of the fibers displayed extensive branching. Closer inspection of an unbranched region (see inset Figure 3) appeared to show bundles of thinner fibers of approximately 10 nm in width. This value is similar to the width of a metal bound trimer of triple helices (Figure 1c). Importantly, in the absence of Fe(II), no peptide assembly or fiber formation was observed, consistent with the DLS observations. It was interesting that with our radial design that fibers were observed. It is possible that the radial assembly of CTHs may lead to a bundle of CTHs that contain sticky ends due to staggering

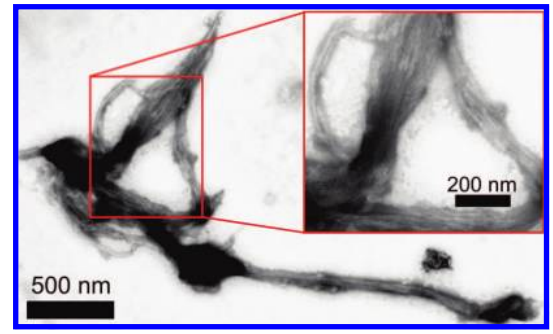

Figure 3. TEM image of **H-byp** (2 mM), Fe(II) (0.3 mM), in HEPES (10 mM pH 7.0).

within the CTHs, and these sticky ends could promote fiber formation. This fiber morphology was found to be lost at higher concentrations of Fe(II) (1 mM) (see Supporting Information), presumably because of the saturation of the bipyridyl ligand with metal ions limiting peptide assembly and fiber growth.

In summary, we have introduced the concept of collagen fiber formation through the radial growth of a collagen mimetic peptide, **H-byp**, via a metal-assisted self-assembling trigger. We demonstrate that the fiber formation was triggered through the addition of Fe(II) and the assembly consisted of branched fibers at concentrations below the ligand−metal binding stoichiometry. This system serves as a proof of principle that collagen fibers can be initiated via nonlinear assembly, and that modifications along the collagen backbone may yield a new class of biologically active fibers. Future studies will investigate how the ligand placement and coordination chemistry can control the architectures of the collagen fibers.

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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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