OL | Organic Letters

Letter

Cite This: Org. Lett. 2019, 21, 5480−5484

pubs.acs.org/OrgLett

Covalent Capture of a Heterotrimeric Collagen Helix

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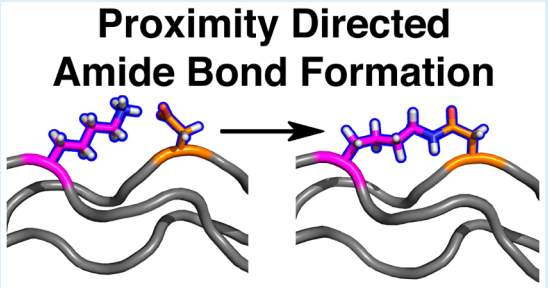
Supporting Information

ABSTRACT: Stabilizing the three-dimensional structure of supramolecular materials can be accomplished through covalent capture of the assembled system. The lysine-aspartate charge pairs designed to direct the self-assembly of a collagen triple helix were subsequently used to covalently capture the helix through proximity-directed amide bond formation using EDC/HOBT activation. The triple helix thus stabilized maintains its folded structure and can now be used for applications previously inaccessible due to problematic folding equilibria.



Self-assembly is used by Nature to create a huge diversity of precise three-dimensional structures ranging from nanofibrous collagen to cell membranes and ribosomes. These processes utilize a large number of noncovalent interactions such as hydrogen bonding, electrostatics, and the hydrophobic effect to drive their assembly. While individually weak, in combination large numbers of these interactions allow reversibility and dynamic response to the environment as necessary. When greater toughness and stability are needed, Nature turns to covalent stabilization of these structures. Noncovalent self-assembly first sets the intricate structure, and subsequently formed covalent bonds stabilize it. This is called covalent capture. This two-step synthesis methodology, self-assembly followed by covalent capture, is a powerful approach to highly stable and molecularly controlled architectures. For the same reasons, this blueprint provided by Nature is an attractive one for chemists to emulate.[1−4] For example, self-assembled complexes have only limited applicability when their concentration is very low (such as an intravenous injection) because under these conditions, equilibrium will favor the disassembled components. Covalent stabilization of these assemblies could eliminate these problems.

Recent advances in our understanding of collagen triple helix assembly and stability has allowed the design of sophisticated structures and applications including controlled assembly of triple helices,[5−9] targeting and imaging of natural collagens,[10−14] and synthetic collagen-based biomaterials.[15−18] Natural collagens are common, being the most abundant protein by mass in humans, primarily in the extracellular matrix.[19,20] The underlying fundamental structural motif, the collagen triple helix, is also found in bacteria, viruses, fungi, and the stalk region of a variety of immune related proteins such as Complement C1q, Ficolin, and Mannose Binding Lectins.[21−25]

The ubiquity of the collagen triple helix suggests a wide range of applications for materials that can mimic, target, or modify it. The collagen triple helix is characterized by the three amino acid repeat $(Xaa\text{-}Yaa\text{-}Gly)_n$ in each of its three peptides. In humans, this sequence is dominated by proline in the Xaa position and hydroxyproline in the Yaa position, but other amino acids may occupy these positions, and common substitutions include negatively charged amino acids in the Xaa position and positively charged amino acids in the Yaa position.[26−28] While homotrimer design and assembly are relatively straightforward, heterotrimer self-assembly is substantially complicated by the large number of possible assemblies.[29,30] For example, a heterotrimer composed of peptides A and B has eight possible canonical assemblies including AAA, BBB, AAB, ABA, BAA, ABB, BAB, and BBA. Our group has described methods utilizing charge paired hydrogen bonds within the triple helix to direct the assembly of specific heterotrimers and has successfully demonstrated both A_2B and ABC heterotrimer self-assembly.[8,9,29,31] Several other groups have also utilized related designs to successfully assemble heterotrimers.[32−35] However, even in well-designed triple helices a substantial population of monomers exists making application for these materials at low concentration problematic where equilibrium will favor the monomer. Similarly, the kinetics of folding for triple helices is notoriously slow, especially for heterotrimers where folding times on the order of weeks is common.[36−38] One solution to both of these problems is to covalently tether the peptide strands to one another eliminating the problem of monomer/trimer equilibrium and accelerating the rate of folding. Covalent linkages

Received: May 20, 2019
Published: June 27, 2019

DOI: 10.1021/acs.orglett.9b01771
Org. Lett. 2019, 21, 5480−5484

have been successfully incorporated during the synthesis of the peptides using bonds at either the N- or C-termini of the peptides.[39−41] Other groups have utilized disulfide bond or oxime ligation strategies to covalently cross-link collagen-like peptide strands in a triple helix.[42−45] However, these synthetic methods are relatively cumbersome and do not have a solution for the control of heterotrimer structure. Here we describe a simple 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) based method where the same charged residues which control the supramolecular assembly, lysine and aspartate, can be readily connected through the formation of an amide bond after self-assembly which maintains the desired triple helical structure against unfolding at low concentration.

We first prepared two peptides, A and B, designed to self-assemble into an AAB triple helix as shown in Figure 1a. The

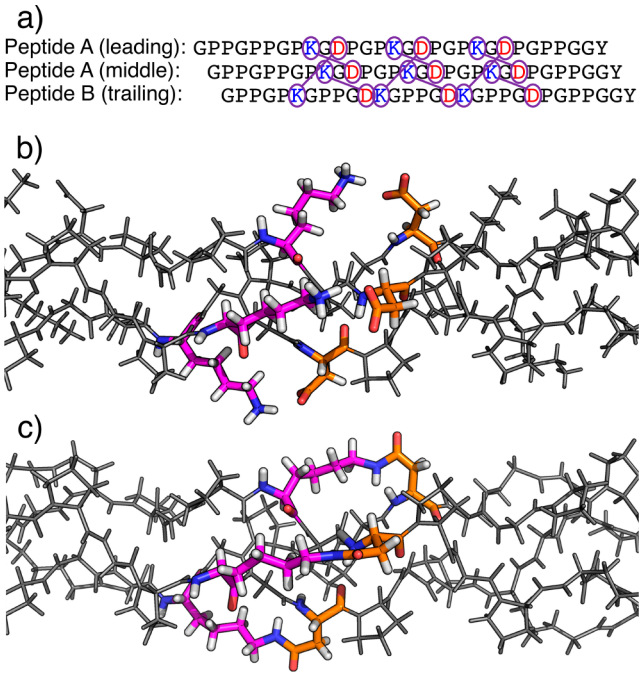


Figure 1. (a) Triple helix sequence design showing potential axial charge paired hydrogen bonds. Model of triple helix showing lysine and aspartate residues as (b) the supramolecular interactions and (c) after amide bond formation.

peptides are N-terminally acetylated and C-terminally amidated to avoid alternative charge stabilization and C-terminally tagged with tyrosine for accurate determination of concentration. When successfully assembled into the AAB register, the triple helix is stabilized by the formation of nine lysine-aspartate axial charge pairs. Competing species (homotrimers and alternative heterotrimers) will have fewer opportunities for stabilization. The two peptides were examined individually as well as in a 2:1 molar ratio by circular dichroism (CD) to observe their triple-helical character and thermal stability as shown in Figure 2a,b and Supporting Information Figure S6. The A_2B mixture resulted in the highest molar residual ellipticity (MRE) indicating the largest fraction folded. It also has the highest melting point (36.0 °C) indicating the best thermal stability, as expected by our design. NMR analysis of the A_2B helix indicates that the designed AAB register is indeed formed (Figures S3−S5).[9,13,29,31,46−51]

A characteristic feature of collagen folding is its rate, which can be very slow. This complicates many analytical methods as well as various potential applications for collagen-like helices. This can be easily observed in the hysteresis displayed between unfolding and folding at equal rates of heating and cooling (10 °C/h). Figure 2b clearly shows slow and incomplete folding even at this slow rate. In fact, several days are required for the A_2B system to regain its maximum MRE value.

Size exclusion chromatography (SEC) is another method which can allow assessment of folding and assembly state. Because this chromatographic method can be performed under native conditions and separates analytes by size, it can discriminate between the folded triple helix and unfolded monomer. Figure 2c shows the SEC of a well folded A_2B mixture in blue where one can see both the triple helix and the monomer. If the A_2B solution is heated to 85 °C, cooled to room temperature, and then injected onto the SEC, one can see in Figure 2c (in red) that the triple helical peak is eliminated leaving only monomers. The SEC shows that even this well folded system still contains monomer species, thus the need for covalent stabilization of the triple helix.

To solve these problems, we performed covalent capture in water using EDC and 1-hydroxybenzotriazole (HoBt) as the activating reagents. Peptide samples were prepared as a 3 mM solution to avoid potential interhelix amide bond formation caused by high concentration. Details of the reaction conditions can be found in the Supporting Information. The reaction was followed by MALDI-TOF MS over time from 1 h to 4 days. Figure 3a shows the full mass spectra at 24 h of reaction, and one can clearly see the peaks of the monomer, dimer, and trimer (blue). Following the covalently captured sample over time, the dimer region remains relatively unchanged and shows two species with masses corresponding to the formation of one or two amide bonds (Figure 3b). Careful examination of the trimer region at different times indicates that a variable number of the lysine-aspartate residues are cross-linked. As seen in Figure 3c, at 1 h little trimer can be observed, and the primary species visible has lost a mass consistent with the formation of two side-chain amide bonds, the minimum number to cross-link the triple helix. As time passes, the number of new amide bonds formed and the intensity of the trimer peaks increase. After 4 days of reaction, the most intense peak corresponds to a triple helix with six new amide bonds (Figure 3d). However, a distribution of masses can be observed indicating three to nine amide bonds formed. This shows that after extended reaction, it is possible to capture all nine charge pairs initially formed in the triple helix.

To demonstrate that this is indeed covalent capture through proximity-directed amide bond formation and not simply the result of amide bonds forming between dissociated monomers, we performed the reaction under identical conditions, but at 50 °C where CD shows the triple helix has dissociated to a monomer. Raising the temperature of a reaction should typically lead to an increase in the reaction rate. In contrast, however, the higher temperature reaction resulted in no formation of dimer or trimer species as seen in Supporting Information Figure S7. This simple test demonstrates the requirement of folding for the success of these reactions.

After covalent capture, the A_2B solution can be analyzed by SEC, and both triple helical and monomer peaks are still visible (Supporting Figure S8). Now, however, the triple helix peak can be collected, and upon reinjection, only a triple helix is observed since it is unable to equilibrate to a monomer. The

DOI: 10.1021/acs.orglett.9b01771
Org. Lett. 2019, 21, 5480−5484

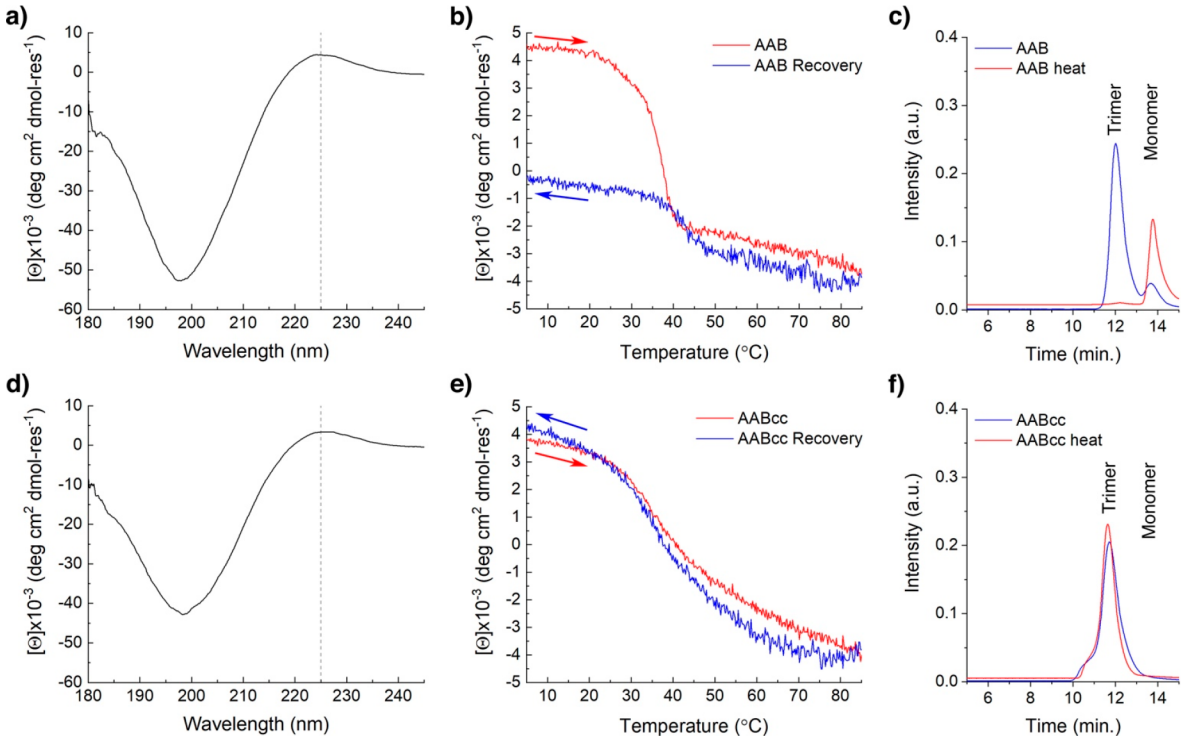


Figure 2. Examination of peptide folding by CD and SEC. (a) CD spectra of a mixture at a 2:1 molar ratio of A:B shows the triple helix maximum at 225 nm. (b) CD melting curve produced by monitoring ellipticity at 225 nm as temperature is increased, and CD refolding curve as temperature is decreased. (c) Size exclusion chromatography of the A_2B triple helix and the same solution after denaturing heating. (d) CD spectra of covalently captured A_2B. (e) CD melting and refolding curves of covalently captured A_2B. (f) SEC of covalently captured A_2B with and without heating.

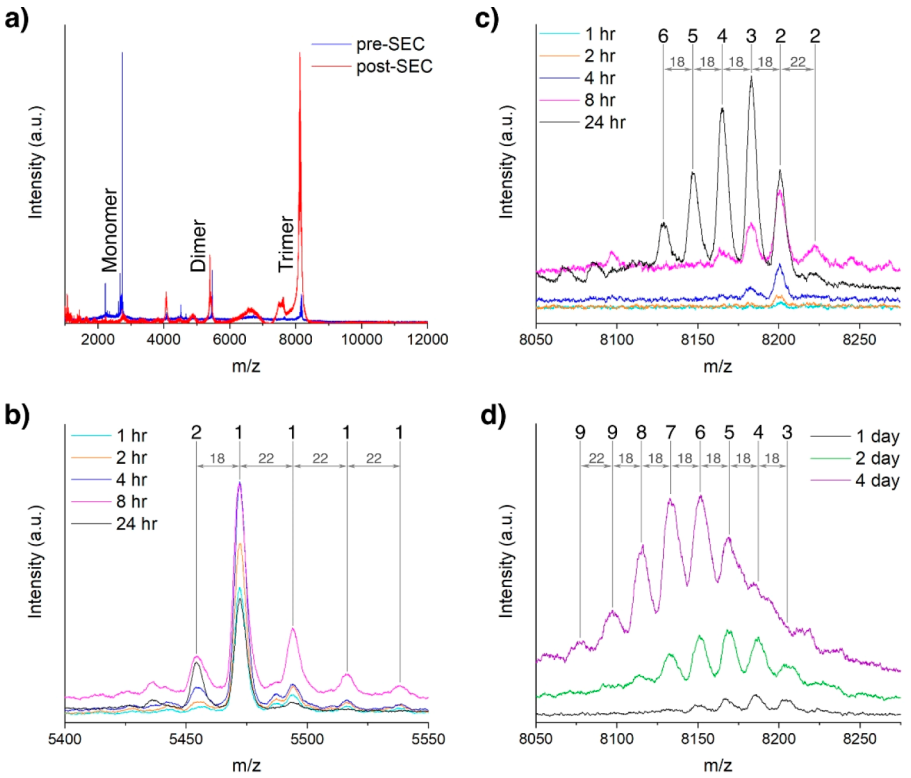


Figure 3. MALDI-TOF mass spectra of the covalently captured A_2B triple helix. (a) Full spectra of the covalent capture mixture after 24 h showing monomer, dimer and trimer (blue). After isolating the triple helical peak by SEC, the mass of the trimer is the most abundant (red). (b) Spectra of the dimer region showing formation of one or two amide bonds. (c) Spectra of the trimer region from 1 to 24 h showing the evolution from two amide bonds to a maximum of six. (d) After 4 days the maximum possible amide bonds, nine, can be observed with six being the most abundant.

DOI: 10.1021/acs.orglett.9b01771
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small change in elution time from the SEC column suggests that the triple helix has become slightly more rigid, but analysis by CD (Figure 2a,d) demonstrates that the triple helical conformation has not been significantly altered as the critical peak at 225 nm is maintained. This covalently captured triple helix can be heated to 85 °C and analyzed by SEC, but now only a triple helix is observed. This demonstrates the helix's effective immunity to thermal denaturation (Figure 2f). Additionally, the unfolding/refolding curve (Figure 2e) displays effectively no hysteresis, demonstrating that the covalent bonds between side chains serve to dramatically accelerate folding. Even when the heating and cooling rates are increased to 60 °C/h, hysteresis of the folding of the covalently captured triple helix is minimal (Supporting Information Figure S9).

The method described here for collagen triple helices is particularly attractive because it makes use of the same stabilizing interactions that drive the self-assembly of a specific composition and register of a triple helix for the covalent capture and also uses common reaction conditions. A similar approach should allow effective use of synthetic collagen mimics in applications that require use at low concentration or where equilibration with other species would become problematic. For example, a covalent capture region attached to a biological sequence of interest could be employed to stabilize and purify the desired collagen mimetic peptide for the activation or inhibition of collagen-binding receptors and other biomaterials. Even with a variable number of amide bonds formed in this covalent capture region, the triple helix will be purifiable and contain the stabilized triple-helical fold for the region of interest. In principle, these materials could be used as injectables despite existing at near zero concentration in the bloodstream.

In summary, we describe a method of covalently capturing the collagen triple helix. Using MALDI-TOF MS, we followed the formation of covalent bonds between lysine and aspartate residues over time. The secondary structure remains triple helical in shape, even with the formation of these covalent bonds, as shown in the CD spectra. Furthermore, the refolding capability is greatly enhanced as seen in the CD refolding curve. This technique allows for a single register trimer that can be purified for use in other applications, an essential tool for the study of collagen and the development of biomaterials and other biomedical applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b01771.

Discussion of peptide design, experimental methods, NMR, additional mass spectra, and circular dichroism (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from the Welch Foundation (C1557) and the NSF (CHE 1709631). I.L. was supported by the Stauffer-Rothrock Fellowship. A.A.J. was supported by the Newton International Fellowship (NF140721).

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