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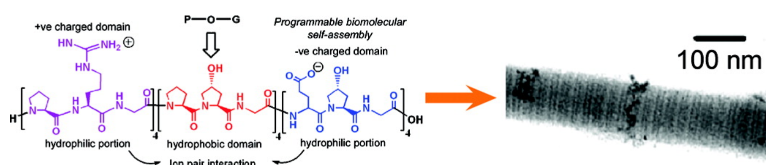

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D-Periodic Collagen-Mimetic Microfibers

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Abstract: Self-assembling peptides have been previously designed that assemble into macroscopic membranes, nanotapes, and filaments through electrostatic interactions. However, the formation of highly ordered collagen-like fibrils, which display D-periodic features, has yet to be achieved. In this report, we describe for the first time a synthetic peptide system that self-assembles into a fibrous structure with well-defined periodicity that can be visualized by transmission electron microscopy (TEM). Specifically, we designed and synthesized a peptide that utilizes charged amino acids within the ubiquitous Xaa-Yaa-Gly triad sequence to bias the self-assembly into collagen-like homotrimeric helices that are capable of fibrillogenesis with the production of D-periodic microfibers. Potential molecular mechanisms for peptide assembly into triple-helical protomers and their subsequent organization into structurally defined, linear assemblies were explored through molecular dynamics (MD) simulations. The formation of thermodynamically stable complexes was attributed to the presence of strong electrostatic and hydrogen bond interactions at staggered positions along the linear assembly. This unexpected mimicry of native collagen structure using a relatively simple oligopeptide sequence establishes new opportunities for engineering linear assemblies with highly ordered nano- and microscale periodic features. In turn, the capacity to precisely design periodic elements into an assembly that faithfully reproduces these features over large length scales may facilitate the fabrication of ordered two- and three-dimensional fiber networks containing oriented biologically, chemically, or optically active elements.

Introduction

The oriented axial assembly of supercoiled protein motifs represents the primary structural feature associated with the load-bearing properties of native protein-based fibrils such as collagen and α-keratin.[1−3] As a principal building block or "protomer", supercoiled motifs assemble into supramolecular fibrils and macroscopic fibers through specific molecular recognition that occurs at the interfaces between these elements, such that linear propagation occurs along the incipient fibril axis.[4−6] The structural features that guide self-assembly are encoded within the sequences of the peptide subunits and include complementary packing of side chains at the interface, as well as electrostatic, hydrophobic, and hydrogen-bonding interactions. Given the richness of the molecular-scale information programmed within proteins, it should not be surprising that synthetic materials have yet to recapitulate the self-assembly behavior of native protein-based, multistranded helical assemblies. Thus, elucidating the design principles that underlie the self-assembly of fibrillogenic proteins into structurally defined supramolecular structures is of interest not only in generating synthetic polypeptides that mimic native structural proteins but also for the design of new materials with biological, chemical, and mechanical properties that exceed those of currently available synthetic polymers.

The triple-helical domain of native collagen comprises a strict recurrence of tripeptide repeat sequence Xaa-Yaa-Gly and displays precise registration of individual amino acids in adjacent helices within a defined oligomerization state imparting structural specificity and fibril-forming properties. Proline (Pro) and (4R)-hydroxyproline (Hyp) occupy the Xaa and Yaa positions, respectively, of the triplet repeats at the highest level of statistical frequency within native collagen sequences. The high imino acid content of collagen sequences is necessary for the stabilization of its triple-helical structure, although alternative substitutions are often observed in native collagen sequences and may be necessary to fully define its biological role. We believe that

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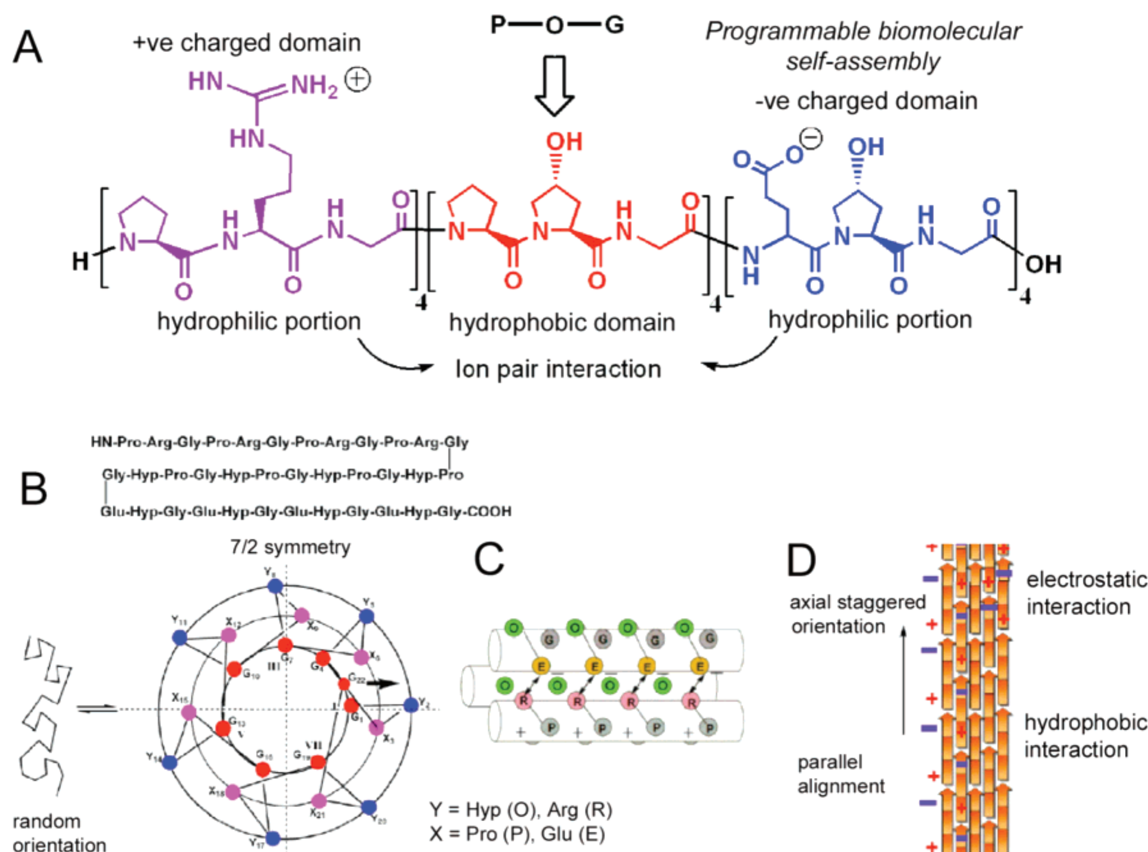

Figure 1. Collagen-mimetic peptide, triple-helical protomer, and fibril assembly. (A) Amino acid sequence of synthetic collagen-mimetic peptide **CPII** indicating the distinct domain structure of collagen triads. (B) Proposed triple-helical wheel diagram of **CPII**, a synthetic collagen-like peptide sequence, adopting 7/2 superhelical symmetry. (C) Side view illustrating proposed interhelical electrostatic interactions driving higher order assembly of a **CPII** homotrimer to yield triple-helical protomers. (D) Schematic representation of **CPII** fibrillogenesis through axially staggered assembly between oppositely charged N- and C-terminal domains of triple-helical peptide protomers.

the synthesis of collagen-mimetic triple helix peptide protomers (THPs) that display the capacity to form triple helices with improved stability and that exhibit a propensity to form linear assemblies through a process of axially oriented alignment will prove to have a number of important practical applications in the design of novel biomaterials. Moreover, the capacity to mimic collagen's ability to form linear assemblies with precisely defined periodic features would represent an important milestone in the design of ordered two- and three-dimensional fiber networks containing oriented chemically, biologically, or optically active elements.

In this report, we describe the solid-phase synthesis of a collagen-mimetic THP in which the sequence was designed to exhibit linear fibril growth upon assembly of the triple-helical structure. In addition, molecular dynamics (MD) simulations were used to assess potential molecular mechanisms for peptide assembly into triple-helical protomers and their subsequent organization into structurally defined, linear assemblies. Sequence specific peptides were generated comprised of three different Xaa-Yaa-Gly domains, including a central core of Pro-Hyp-Gly repeat sequences flanked by distinct sets of peptide repeats containing either negatively (Glu) or positively (Arg) charged amino acid residues (Figure 1). The Pro-Hyp-Gly peptide sequence forms the structurally critical hydrophobic core of the assembly, which is responsible for maintaining the thermodynamic stability of the collagen triple-helical structure. If positioned appropriately at the N- and C-terminal portions of the THP, MD simulations in association with transmission electron microscopy demonstrated that the electrostatic interactions of the charged residues (Arg and Glu) facilitated a preferential linear oligomerization of protomers within fibrils and promoted a staggered orientation between adjacent triple helices that was similar to that observed in native fibrous collagens. Likewise, MD simulation illustrated that linear oligomerization was reinforced by hydrogen bonds formed within the N- and C-terminal interface of adjacent THPs. Self-assembling peptides have been previously designed that assemble into macroscopic membranes, nanotapes, and filaments through electrostatic interactions.[7−9] However, the peptide described herein is the first to yield triple-helical protomers that undergo collagen-like fibrillogenesis through complementary electrostatic interactions that are reinforced by hydrogen bond formation. Significantly, synthetic *D-periodic* collagen-mimetic fibers have been generated through the spontaneous self-assembly of short triple-helical oligopeptides, which heretofore has not been previously achieved.

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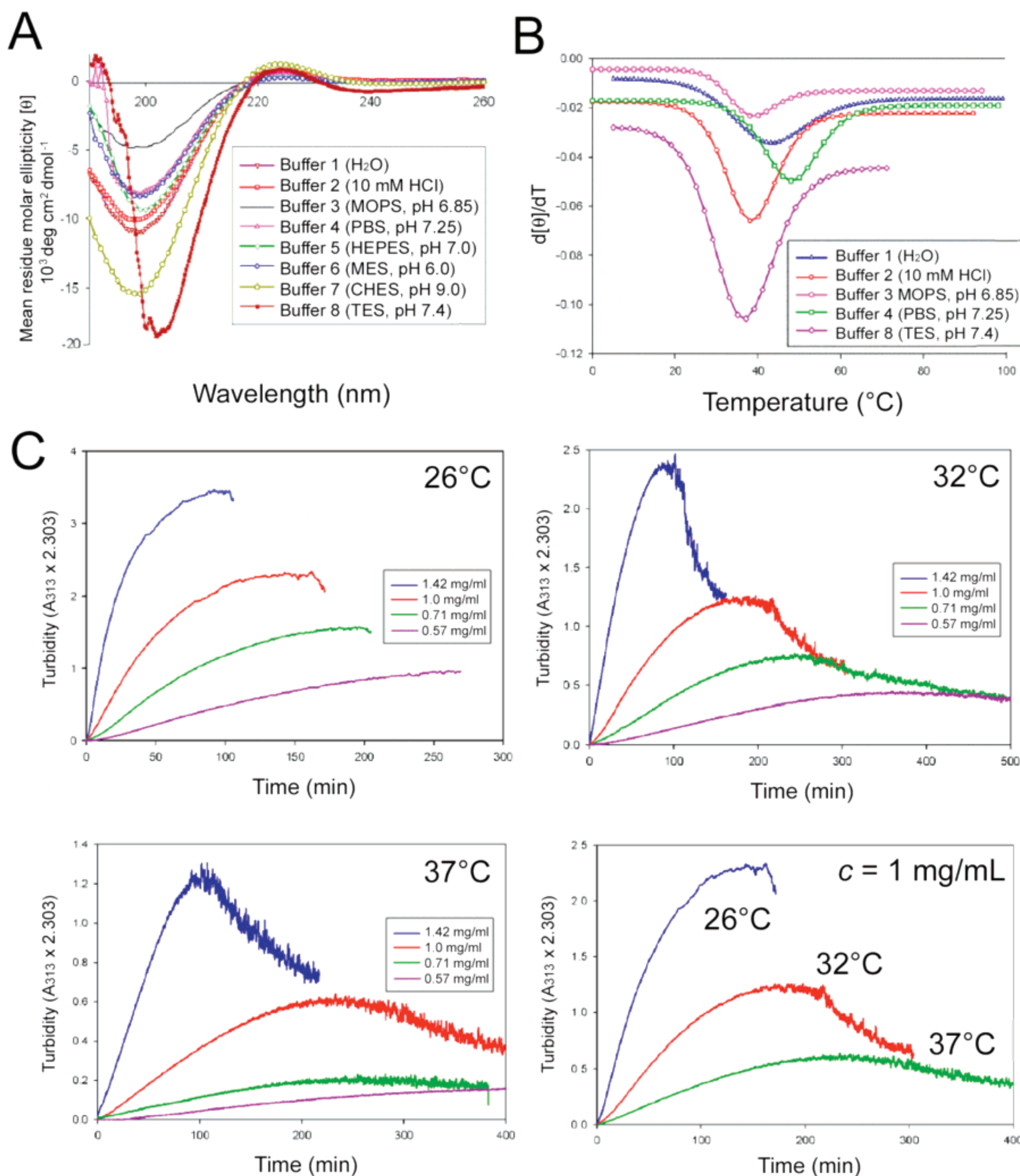

Figure 2. Circular dichroism spectroscopy and turbidimetric analysis of CPII collagen-mimetic peptides. (A) CD spectroscopy of collagen-mimetic peptide **CPII** (0.5 mg/mL). Mean residue molar ellipticity [θ] 10 deg^3 cm^2 dmol^{-1} as a function of solvent system at 4 °C. (B) First derivative plots, d[θ]/dT, of thermal denaturation profiles for **CPII** in varying solvent systems. Buffer 1: H_2O, Buffer 2: 10 mM HCl, Buffer 3: 10 mM MOPS (pH 6.85), Buffer 4: PBS (pH 7.25), Buffer 5: 10 mM MES (pH 6.0), Buffer 6: 10 mM HEPES (pH 7.0), Buffer 7: 10 mM CHES (pH 9.0). Buffer 8: 30 mM mM Na_2HPO_4, 30 mM TES, 135 mM NaCl (ionic strength 0.225), pH 7.4. (C) Turbidity profiles for collagen-mimetic peptide **CPII** determined at concentrations between 0.57 and 1.42 mg/mL and temperatures between 26 and 37 °C. Absorbance was acquired at 313 nm as a function of time and turbidity presented as $A_{313} \times$ 2.303.

Results

Determination of Triple-Helical Conformation of CPII. The collagen-mimetic peptide, NH-(Pro-Arg-Gly)$_4$-(Pro-Hyp-Gly)$_4$-(Glu-Hyp-Gly)$_4$-COOH, **CPII**, was synthesized by a solid-phase synthetic strategy (Supporting Information). Circular dichroism (CD) spectroscopy was performed to ascertain the triple-helical character of the synthesized peptide in various buffer solutions, as well as related thermal transition curves (Figure 2A). CD spectra in various buffer solutions exhibited a positive peak at 221−222 nm, a crossover peak at 213−214 nm, and a negative peak at 197−198 nm. These spectral positions are very similar to those of native collagen and collagen-mimetic peptide assemblies and, therefore, are consistent with the triple-helical conformation through homotrimer formation.

Thermal unfolding studies were also performed to confirm the presence of triple-helical conformation of **CPII** in various buffer solutions (Figure 2B). Thermal denaturation demonstrated melting temperatures (T_m) ranging from 37 to 48 °C. The highest T_m was noted in PBS, and the lowest was noted in a buffer solution of 30 mM Na_2HPO_4, 30 mM TES, 135 mM NaCl (ionic

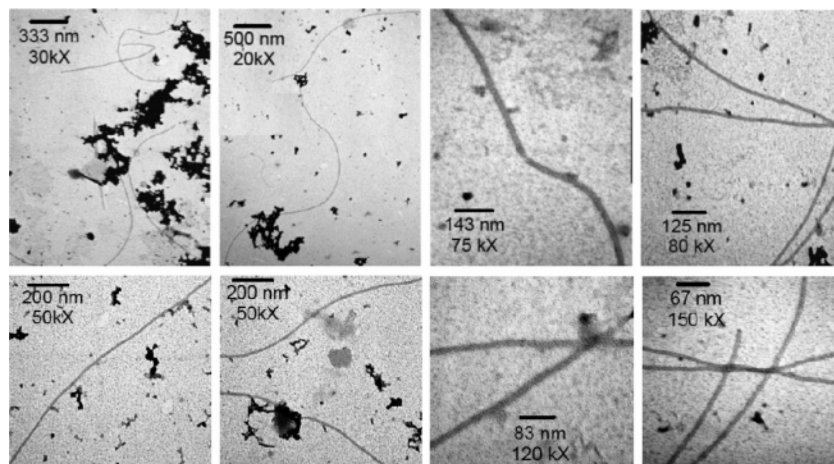

Figure 3. Fiber formation from CPII collagen-mimetic peptides. Transmission electron micrographs of collagen-mimetic peptide fibril formation in the absence of thermal annealing of **CPII**. The final buffer composition was 30 mM TES, 30 mM phosphate, 135 mM NaCl at a **CPII** concentration of 0.5 mg/mL. Nonbanded fibers with average lengths of 3.0−4.0 μm and diameters of 12−15 nm were observed.

strength 0.225), pH 7.4. The latter is similar to the reported melting temperature of ca. 40 °C for monomeric type I collagen in a similar buffer solution (Supporting Information).[10]

Turbidity Analysis of Peptide Assembly in Solution. As an initial measure of fibrillogenesis, turbidity profiles were determined for **CPII** at varying peptide concentrations and solution temperatures in 30 mM TES, 30 mM phosphate, and 135 mM NaCl after initial "thermal annealing" by heating the peptide solution to 75 °C for 40 min followed by cooling to room temperature (Figure 2C). Similar to collagen fibrillogenesis, growth phases were consistent with a first-order rate process that required a minimum peptide concentration to trigger peptide self-assembly. However, a number of features of **CPII** aggregation were distinct from collagen fibrillogenesis, including shorter lag phases and a decline in turbidity after an initial plateau. The latter phenomenon is consistent with sedimentation of **CPII** peptide aggregates, which, unlike monomeric collagen, do not form a physical network or gel. In addition, we observed that the growth rate for **CPII** self-assembly decreased with increasing temperature. Leikin et al.[11] and others[10,12] have suggested that fibrillogenesis is favored as the temperature approaches the T_m of collagen due to the release of structured water, increasing mobility of side chains, and loosening of the triple helix, all of which may promote structural interactions that drive collagen self-assembly. However, Glu-Hyp-Gly guest triplets may decrease the propensity toward triple-helix formation in (Pro-Hyp-Gly) containing host peptides.[13] Thus, given the relatively small size of **CPII** compared to collagen, local unfolding of the negatively charged C-terminal domain with increasing temperature may limit the thermodynamic stability of self-assembled structures. As a consequence, although the natural logarithm of the rate constants was linearly dependent on $1/T$, obeying the Arrhenius relationship, the calculated activation energy for the growth phase of **CPII** was negative (-74 kJ mol^{-1}).

TEM Analysis of CPII Fibril and Microfiber Formation. **CPII** self-assembly, initiated as described for turbidity studies, was examined at varying time intervals by transmission electron microscopy. In the absence of thermal annealing nonbanded fibers with average length of 3−4 μm and diameters of 12−15 nm were observed (Figure 3).

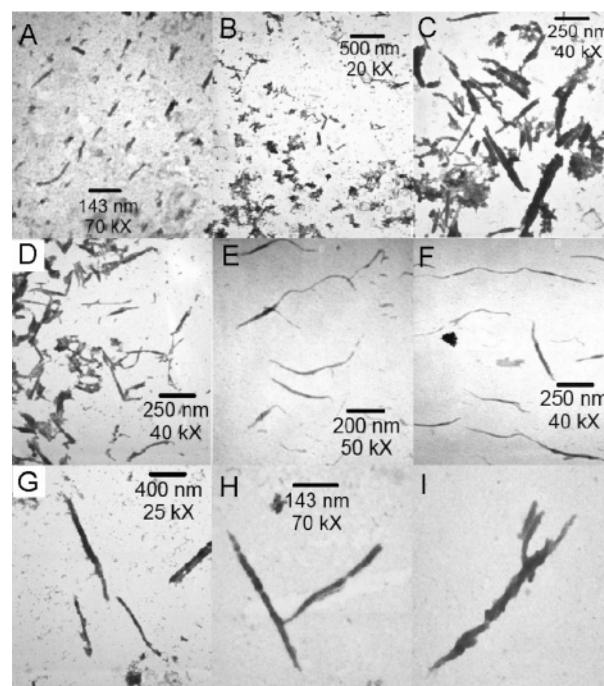

Figure 4. Formation of collagen-mimetic fibrils. Transmission electron micrographs of collagen-mimetic peptide fibril formation after a 4 h (A−C) or 2 day (D−I) incubation period in fibril forming buffer at room temperature. Final peptide concentration was 0.71 mg/mL in 30 mM TES, 30 mM Na_2HPO_4 dibasic heptahydrate, 135 mM NaCl. The peptide solution was initially heated to 75 °C for 40 min and then cooled to 23 °C. The length of tapered segments first noted at 4 h increases over 48 h and suggests that the growth of the linear aggregates occurs by the successive addition to the ends of the fibrils.

However, after thermally annealing solutions of **CPII**, fiber growth proceeded within several hours by initial formation of smooth fibrils that were hundreds of nanometers in length and tens of nanometers in diameter (Figure 4). These fibrils displayed tapered tips similar to the tactoidal ends of native collagen fibers from which continued fiber growth is thought to occur.

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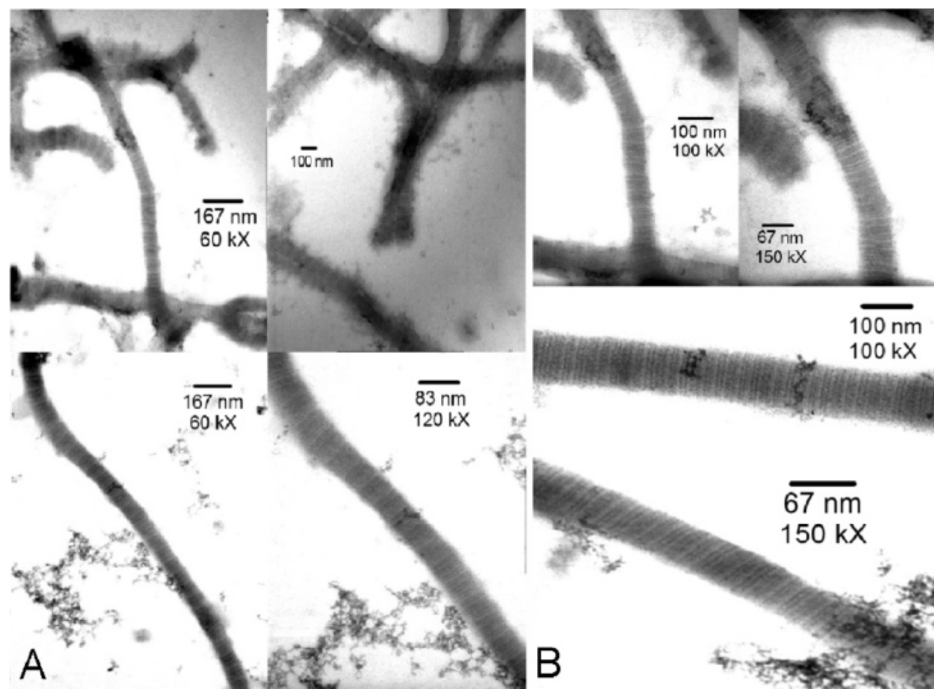

Figure 5. Formation of D-periodic collagen-mimetic fibers. (A) Transmission electron micrographs of uranyl acetate-stained fibers of collagen-mimetic peptide **CPII** formed after a 9 day incubation period at room temperature. (B) High magnification transmission electron micrographs reveal well-defined D-periodic structure.

Samples examined after longer incubation periods demonstrated the formation of uniform micron-length fibers that were approximately 70 nm in diameter with well-defined transversely banded structure akin to the D-periodicity of collagen (Figure 5). Analysis of high magnification images revealed that the length of D-periodic gap and overlap regions was approximately 18 nm. Of note, fibril formation did not occur in the absence of salt (e.g., water or 10 mM HCl) and at high phosphate concentrations (i.e., 60 mM Na_2HPO_4), only nonbanded, poorly formed linear aggregates were noted.

Molecular Simulations of CPII Triple-Helical Protomers and Higher Order Assembles. Molecular dynamics simulations were performed to examine potential structural features that contribute to the assembly of **CPII** THPs, as well as to the formation of axially oriented staggered protomer−protomer assemblies. A system of individual **CPII** peptides, in parallel or antiparallel orientation, reached an energy minimum after 5 ns in a NpT dynamics simulation at 23 °C and 1 atm (Figure 6 and Supporting Information). A thermodynamically stable, close-packed, triple-helical protomer was observed for both systems, and the presence of a significant number of hydrogen bonds contributed to their thermodynamic stability. Although the distance matrix and the number of hydrogen bonds between the peptides were similar for both THP systems, we observed a significant difference in the distribution of electrostatic potential with strong positive and negative potentials noted at the termini of THPs produced from parallel oriented **CPII** peptides. Significantly, subsequent simulations of this system yielded a linear assembly of head-to-tail, axially oriented protomers that was promoted by the presence of substantial electrostatic and hydrogen bonding interactions, which were much more favored than THPs generated from antiparallel oriented **CPII** peptides.

Additional simulations were conducted to investigate whether these linear peptide assemblies display a propensity for further fibrillogenesis-like growth. Specifically, we simulated the interactions of three linear complexes, each produced from four THPs that were initially assembled from three **CPII** peptides in parallel orientation. After a 10 ns NpT molecular dynamics simulation at 23 °C and 1 atm, an energy minimum was achieved with formation of thermodynamically stable staggered complexes attributed to strong electrostatic effects at the N- and C-termini of THPs and hydrogen bond interactions between THPs arising from parallel oriented peptides. Indeed, in a manner similar to collagen self-assembly, these linear fibril-like structures displayed staggered alternating high and low charge density domains. In summary, both the experimental data and the results of molecular simulations suggest that staggered complex formation of higher order THP multimers gives rise to the observed D-periodic collagen-mimetic microfibers (Figure 7).

Discussion

The association of three peptide chains that interact to form triple-helical protomers initiates collagen supramolecular structures. Native protomers have at least one triple-helical collagenous domain and two noncollagenous domains of variable sequence and size that are positioned at the N- and C-termini.[14] Distinct supramolecular structures are formed by the oligomerization of protomers through interactions that involve the formation of end-to-end connections, lateral associations, and supercoiling of helices. However, the critical protomer−protomer recognition steps that result in assembly of collagen into a fibril or fiber network remain poorly defined.

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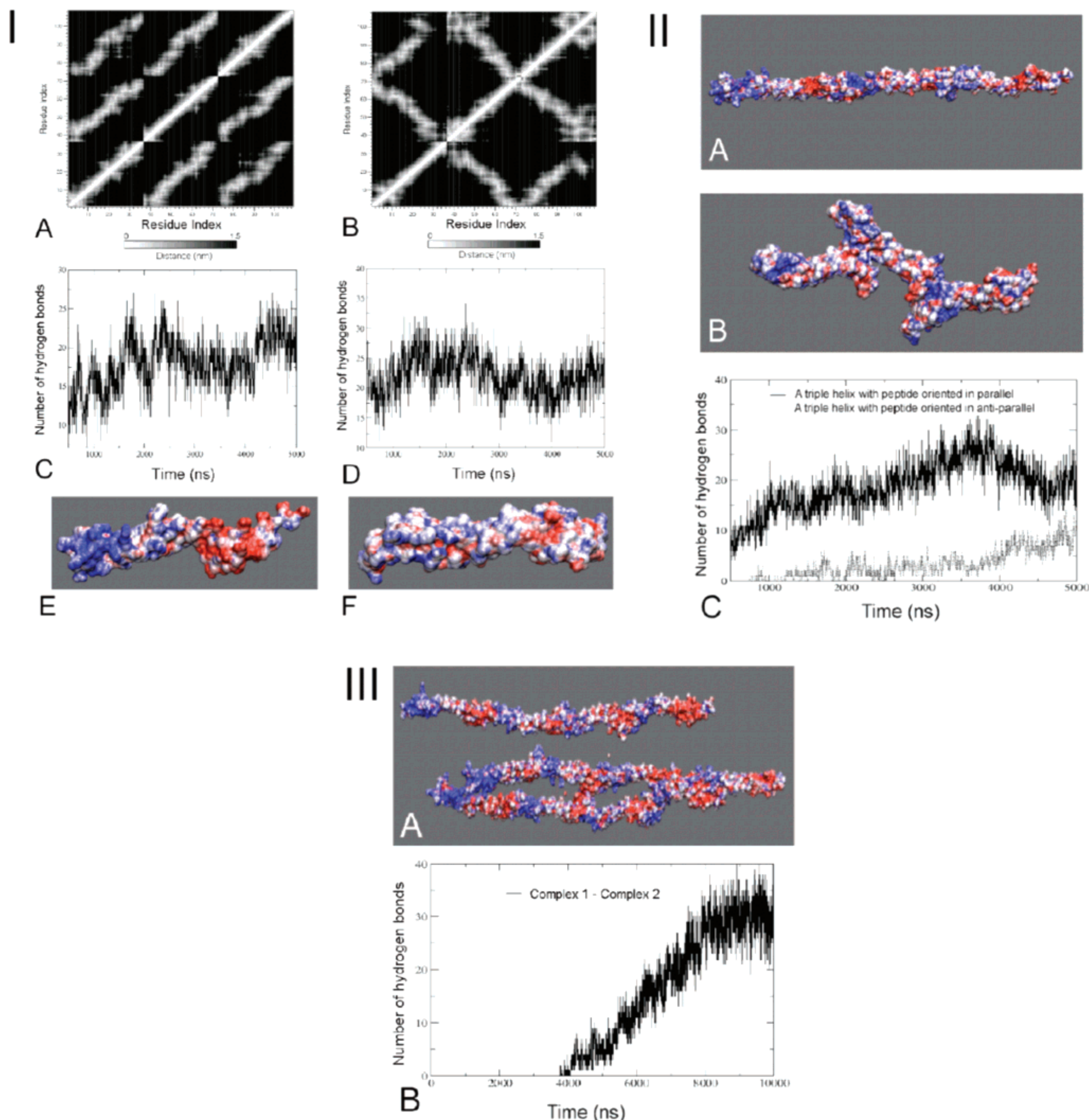

Figure 6. Molecular dynamic simulations of CPII triple-helical protomers and protomer assemblies. (I) Distance matrix (A, B), number of hydrogen bonds (C, D), and electrostatic potential on the solvent accessible surface (blue: positive, red: negative) (E, F) for a triple-helical protomer when peptides are oriented in parallel or antiparallel, respectively. Peptide 1: residue 1−36, peptide 2: residue 37−72, peptide 3: residue 73−108. (II) Electrostatic potential distribution on the solvent accessible surface of an assembly of three triple-helical protomers (blue: positive, red: negative), when THPs are produced from individual peptides in parallel (A) or antiparallel (B) orientation. C Number of hydrogen bonds formed between the triple-helical protomers assemblies. (III) (A) Electrostatic potential distribution on the solvent accessible surface of three complexes, each formed from four triple-helical protomers produced from parallel oriented **CPII** peptides (blue: positive, red: negative). (B) Number of hydrogen bonds formed between the three complexes. The images were made with VMD software and Xmgrace software support.

Recently, there have been several reports of collagen-like peptides that self-associate to higher molecular weight linear structures through the presence of chemical moieties at the N- and C-termini that promote the formation of cysteine knots,[15] facilitate native chemical ligation,[16] or lead to noncovalent aromatic-stacking interactions.[17] Thus far, none of these systems have yielded D-periodic microfibers that are characteristic of collagen. In this report, we designed and synthesized a peptide, **CPII**, that utilizes charged amino acids within the ubiquitous Xaa-Yaa-Gly triad sequence to bias the self-assembly into collagen-like homotrimeric helices that are capable of fibrillogenesis with the production of D-periodic microfibers.

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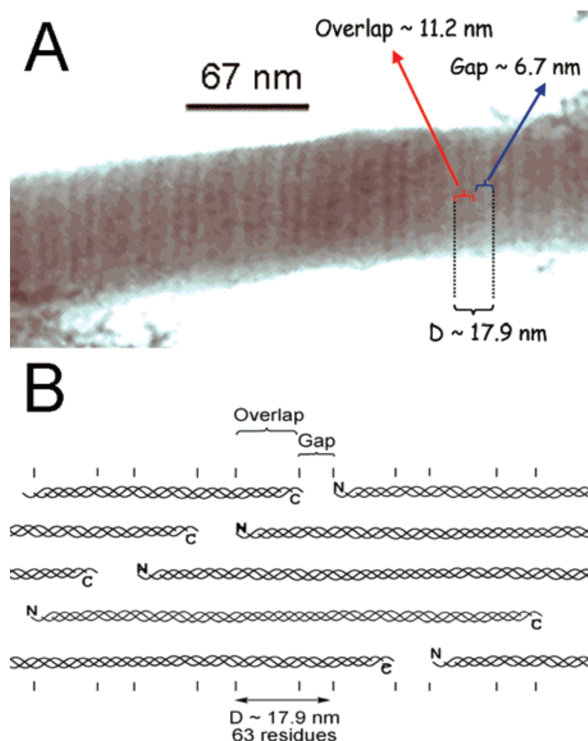

Figure 7. Schematic representation of the staggered relationship between self-assembled THP oligomers and the observed D-periodic banding pattern in collagen-mimetic microfibers. In a staggered array, each "fiber-forming" unit consists of a linear THP oligomer that is axially displaced with respect to its neighbor by a constant distance D (~18 nm) or by an integral multiple of D. Each D-period encompasses a "gap" zone and a larger "overlap" zone. The axial spacing of residues in the THP is 0.286 nm, so that a D-period extends over 63 residues.

Glutamic acid and arginine are the most common charged amino acids found in collagen, and investigations of synthetic peptides have demonstrated that relatively stable triple helices can be produced when these residues are found in the Xaa and Yaa positions of the triplet, respectively.[13] However, differences in triple-helix stability have been noted among a range of evaluated tripeptide repeats that have been introduced into guest sites in collagen-mimetic peptide sequences. These studies have demonstrated that highly stable motifs such as Pro-Hyp-Gly can stabilize the collagen structure such that Glu-Hyp-Gly repetitive triplets, which have a weaker propensity for triple-helix formation, can be incorporated into the collagen sequence.[7,13,18] In the sequence of **CPII**, $(Pro\text{-}Hyp\text{-}Gly)_4$ formed a critical hydrophobic core, which facilitated the capacity of N- and C-terminal $(Pro\text{-}Arg\text{-}Gly)_4$ and $(Glu\text{-}Hyp\text{-}Gly)_4$ domains to reinforce the preferred linear oligomerization within a fibril and promote staggered orientation between adjacent fibrils in the proposed structure of the collagen-mimetic fiber. Of note, **CPII** shows a high propensity for self-association following a nucleation-growth mechanism even at lower concentrations (<1.0 mg/mL) and neutral pH. This observation contrasts sharply with the self-assembly behavior of the $(Pro\text{-}Hyp\text{-}Gly)_{10}$ homotrimer, in which TEM studies indicated the presence of nonspecific aggregates only at much higher concentrations (ca. 7 mg/mL).[12]

The significance of our electrostatically driven approach lies in the observation that self-assembly of this protomer produced D-periodic microfibers that resemble native fibrous collagen. D-periodicity in collagen has its origins in differential intermolecular interactions that occur between protomers, which are mediated either through electrostatic interactions between oppositely charged side chains or through hydrophobic interactions. These interactions are maximized when each protomer is displaced axially with respect to its nearest neighbor.[19,20] In a parallel molecular assembly, the repeated expression of this staggered association generates a D-periodic structure. Each D-period is made up of two zones, one containing higher charge density than the other. This staggered structure of unequal molecular charge density accounts for the characteristic banding pattern of fibrils stained with uranyl salts. Uranyl acetate is only weakly dissociated in aqueous solution and exists as anionic and cationic complexes that bind to charged side chains. The repeating darkly staining transverse bands are thought to be a result of preferential penetration of electron-dense uranyl ions into regions of lowest packing density. The D-periodicity of the synthetic collagen-mimetic microfibers was approximately 18 nm, which, although considerably shorter than the 67 nm D-period observed for native collagen, is longer than one might have anticipated given the 10 nm length of the designed protomer. The banding distance observed for **CPII** assemblies suggests that the "functional" unit for fiber formation may involve oligomers consisting of multiple, laterally associated protomers, such that their staggered assembly defines an arrangement in which overlap zones create periodic regions of varying peptide packing and charge density. Notably, synthetic fibers were initially observed to exhibit pointed tips similar to the tactoidal ends of native collagen fibers from which continued fiber growth is thought to occur. Prockop et al.[21] and others[22,23] have suggested that this distinct morphological feature is consistent with a model for tip growth for native collagen based on the helical packing of aligned and parallel D-staggered molecules. Given this similarity, such an assembly process may be operative in our system.

In summary, our experimental studies show that D-periodic collagen-mimetic fibrils and microfibers, which are very similar to those formed *in vivo*, can be obtained through the linear assembly of a small collagen-mimetic peptide driven through electrostatic interactions. Mechanistically, molecular modeling suggests that staggered axial growth appears to be the preferred higher order structure for **CPII** peptides due to strong electrostatic effects and hydrogen bond formation between parallel oriented peptides assembled as triple-helical protomers. We believe that the synthesis of collagen-mimetic THPs that exhibit a propensity to form linear assemblies through a process of axially oriented alignment will prove to have a number of important practical applications in the design of novel biomaterials. Moreover, the capacity to mimic collagen's ability to form linear assemblies with precisely defined periodic features represents an important milestone in the design of ordered materials for diverse applications in medicine and bionanotechnology.

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Supporting Information Available: All experimental procedures as well as HPLC purification and mass spectroscopic data for **CPII** and additional results for molecular dynamics simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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