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Collagen-Related Peptides: Self-Assembly of Short, Single Strands into a Functional Biomaterial of Micrometer Scale

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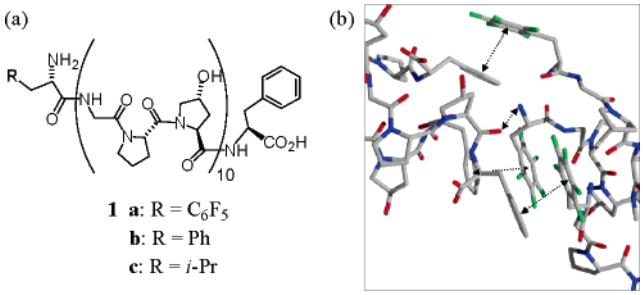
Collagen, the most abundant protein in mammals, has fascinated scientists because of its extraordinary structural features and biological importance. In 1961, Rich and Crick suggested that collagen possesses a triple-helical structure,[1] which was later confirmed by X-ray analysis of 30-mer collagen-related peptides (CRPs), such as (Pro-Hyp-Gly)$_4$Pro-Hyp-Ala-(Pro-Hyp-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$.[2] The large triple-helical domains of collagen consist of three peptide strands with Gly-X-Y repeating motifs, 300 nm in length (vs ~9 nm for CRPs), with the X and Y mainly populated by Pro and Hyp, respectively. In addition, there are short telopeptide regions at the N- and C-termini, which are important for fibril assembly.[3] The rigidity of the ropelike super-helix and the assembled fibril helps provide mechanical strength to tissues, such as skin, tendons, ligaments, and blood vessels. Following vascular injury, the exposed collagen in the vessel wall promotes tissue repair by activating platelets for aggregation and adhesion. However, excessive platelet activity, such as from rupture of an atherosclerotic plaque, can lead to pathological thrombosis with attendant arterial obstruction, as in myocardial infarction or stroke.[4]

The study of the structure, stability, and function of collagen triple helices has been facilitated by the use of synthetic collagen model peptides.[5] Although oligomerized CRPs, via dendrimer assembly or covalent crosslinking, can effectively induce platelet aggregation, less organized CRPs have lacked this property.[6] We sought to identify a *single-stranded CRP* that could spontaneously self-assemble into a bioactive material. Recently, two research groups obtained micrometer-scale CRP-based materials from the self-assembly of covalently attached triple-stranded entities by employing a cysteine knot.[7] We report on the design, synthesis, and characterization of novel single-stranded CRP **1a** (Figure 1a), which self-assembles by noncovalent interactions into a triple-helical, micrometer-scale, composite fibrillar substance with noteworthy biological activity.

The requirement of the telopeptide regions of collagen,[3] and specific Tyr and Phe residues within the C-terminal telopeptide chain,[8] suggests the importance of aromatic residues in the "code" for collagen self-assembly.[8] Inspired by nature, we sought to explore short, single-stranded CRPs that possess the main repeat-unit of collagen, Gly-Pro-Hyp, and contain aromatic recognition units at the N- and C-termini. Phenyl and pentafluorophenyl end-groups were considered, given the strong noncovalent aromatic-stacking interaction between benzene and hexafluorobenzene.[9] In this regard, 32-mer peptide **1a**, with pentafluorophenylalanine (F_5-Phe) and Phe residues at the N- and C-termini, respectively, was of interest. We speculated that interstrand aromatic-stacking and ordered hydrophobic interactions would encourage the usual triple-helical species to associate into longer, higher-order peptide strands that represent collagen-like structures (fibrils and fibers).



1 **a**: R = C_6F_5
b: R = Ph
c: R = *i*-Pr

Figure 1. (a) Structure of peptides **1a**−**c**; (b) modeled interface of two triple helices of **1a** (key aromatic and H-bond interactions noted).

The feasibility of this hypothetical self-assembly process was investigated by computational molecular modeling. The interface between two head-to-tail triple helices of **1a** was probed on a suitable model structure by using an XED (extended electron distribution) force field (Figure 1b).[9a,10] A reported X-ray structure for a CRP[2a] was mutated to incorporate F_5-Phe at the N-terminus (Hyp-position) and Phe at the C-terminus (Gly-position) to construct a starting conformation.[10] As the two triple-helices approached on their central axis, the phenyl/pentafluorophenyl pairs adopted a face-to-face (FTF) orientation resulting in a total interface binding energy of −55.2 kcal/mol.[11] For comparison, the interfaces of analogous CRPs **1b** and **1c** were also examined.[10] In the case of **1b**, a lower interface energy was obtained (total energy of −49.2 kcal/mol) with no symmetrical FTF interactions present.[10] A substantial drop-off in energy occurred with **1c** (total energy of −32.5 kcal/mol).[10] Thus, the possibility of strong interactions between opposite ends of the triple helices of **1a** is supported by the computational analysis.

Peptide **1a** was synthesized by standard FastMoc chemistry, purified by reversed-phase HPLC, and characterized.[10] The material was found to adopt a triple-helix structure by CD spectroscopy (θ_{max} = 225 nm).[10] The thermal stability of **1a** was studied by CD (3 °C increments with 5-min equilibration), and its melting temperature (T_m) is 57 °C (Figure 2).[10] This result was confirmed by a temperature-dependent ^{1}H NMR study, in which we observed a characteristic downfield shift for the δ-H of proline (originally δ 3.0−3.5 ppm) from 55 to 65 °C (with equilibration).[10] Thus, **1a** forms a stable triple helix well above room temperature. It is noteworthy that its thermal stability is slightly higher than that for a recently described collagen-mimetic compound (T_m = 47 °C) with three peptide strands covalently linked by a pair of disulfide bonds.[7b] The lower melting temperature of **1a** compared to 31-mer reference Ac(Gly-Pro-Hyp)$_{10}$GlyOH (**2a**; T_m = 70 °C) may be attributable

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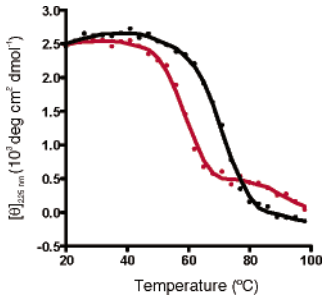

Figure 2. Thermal equilibrium curves of peptide **1a** (red) and reference CRP **2a** (black) in water (0.25 mM) by CD spectroscopy at 225 nm.

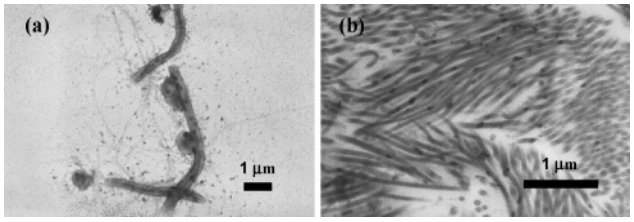

Figure 3. TEM images of (a) self-assembled **1a** (ca. 7 μm in length) and (b) a murine blood vessel cross-section.

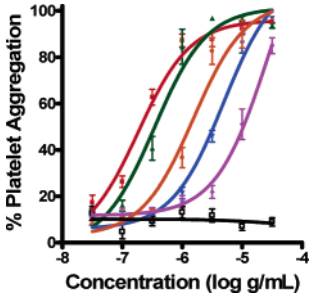

Figure 4. Platelet response to collagen (red square); **1a** in PBS (7 days, green triangle; H+F/7 days, blue triangle; H+F/24 h, purple diamond) and H_2O (24 h, orange circle); and **2b** in PBS (H+F/24 h, open square), by measuring the optical density decrease (650 nm) from $t = 0$ to $t = 5$ min.

to some structural disruptions ("fraying") at the ends of triple-helical **1a** by the phenyl and pentafluorophenyl groups.

Dynamic light scattering (DLS) measurements were conducted to determine the size of the supramolecular assemblies formed by peptides **1a** and **2a** in water (0.5 mg/mL, 25 °C) after heating at 70 °C for 10 min ("H") and passing through a 0.45-μm filter ("F").[10] A fresh solution of **1a** contained two species, sized at 3 and 190 nm, and after 24 h these converged into an aggregate material with an approximate size of 1000 nm. In contrast, reference peptide **2a** showed two species with sizes around 4 and 100 nm, which did not increase over the same time period. These results suggest that the self-association of **1a** is being reinforced by the phenyl−pentafluorophenyl aromatic-stacking mechanism.

We assessed the size and morphology of self-assembled **1a** by transmission electron microscopy (TEM).[10] An aqueous solution of **1a** (0.05 mg/mL) was deposited on a copper grid for imaging (TEM). In every experiment, we observed μm-long, composite fibrils (Figure 3a; av diam = 0.26 μm) that resemble the collagen fibrils found in murine aortic tissue (Figure 3b; av diam = 0.05 μm). The fibril dimensions for **1a** require a combination of end-to-end (linear) and side-to-side (lateral) assembly.

The ability of **1a** to mimic collagen's biological function was evaluated in a human platelet aggregation assay (Figure 4).[10] Some test solutions of **1a** in PBS (pH 7) or water were incubated for 24 h or 7 days (4 °C), and other samples were denatured (H+F), and reannealed at 4 °C. The different solutions of **1a** induced platelet aggregation, but shorter incubations and "H+F" samples showed decreased potency. Remarkably, peptide **1a** (untreated, aged 7 days; EC_{50} = 0.37 μg/mL) was nearly equipotent with equine type I collagen (EC_{50} = 0.25 μg/mL), whereas 30-mer reference peptide **2b** [(Pro-Hyp-Gly)$_{10}$] failed to aggregate platelets.[12] Our findings indicate that **1a** can self-organize over time into aggregates of appropriate length and conformation to meet the structural requirements for platelet recognition (presumably at platelet collagen receptors). In a confirmatory experiment, platelet aggregation induced by a fixed concentration (EC_{60-70}) of **1a** or collagen was inhibited by the GPIIb/IIIa antagonist elarofiban (RWJ-53308).[13]

In conclusion, we have observed the self-assembly of a short (9-nm) single-stranded CRP, **1a**, by noncovalent means, into collagen-like fibrils with collagen-mimetic properties. Notably, micrometer-length, triple-helix-containing, composite fibrils were formed, as determined by CD, DLS, and TEM data. Also, **1a** acted as a functional protein-like material, with an ability to induce platelet aggregation in analogy to collagen. The aromatic−aromatic recognition motif employed in our study offers a straightforward approach to collagen-mimetics and has important implications for the design of triple-helical building blocks that can spontaneously oligomerize into functional fibrillar structures.

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Supporting Information Available: Complete ref 13a, experimental section, CD and NMR spectra, DLS, EM data, and modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rich, A.; Crick, F. H. C. *J. Mol. Biol.* **1961**, *3*, 483−506.
(2) (a) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75−81. (b) Berisio, R.; Vitagliano, L.; Mazzarella, L.; Zagari, A. *Protein Sci.* **2002**, *11*, 262−270. (c) Hyp = 4(*R*)-hydroxy-L-proline.
(3) Helseth, D. L., Jr.; Veis, A. *J. Biol. Chem.* **1981**, *256*, 7118−7128.
(4) (a) Nieswandt, B.; Watson, S. P. *Blood* **2003**, *102*, 449−461. (b) Penz, S.; Reininger, A. J.; Brandl, R.; Goyal, P.; Rabie, T.; Bernlochner, I.; Rother, E.; Goetz, C.; Engelman, B.; Smethurst, P. A.; Ouwehand, W. H.; Farndale, R.; Nieswandt, B.; Siess, W. *FASEB J.* **2005**, *19*, 898−909.
(5) (a) Feng, Y.; Melacini, G.; Taulane, J. P.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10351−10358. (b) Fields, G. B.; Prockop, D. J. *Biopolymers* **1996**, *40*, 345−357 and references therein. (c) Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, *392*, 666−667. (d) Jenkins, C. L.; Raines, R. T. *Nat. Prod. Rep.* **2002**, *19*, 49−59. (e) Shah, N. K.; Ramshaw, J. A. M.; Kirkpatrick, A.; Shah, C.; Brodsky, B. *Biochemistry* **1996**, *35*, 10262−10268.
(6) (a) Rao, G. H. R.; Fields, C. G.; White, J. G.; Fields, G. B. *J. Biol. Chem.* **1994**, *269*, 13899−13903. (b) Morton, L. F.; Hargreaves, P. G.; Farndale, R. W.; Young, R. D.; Barnes, M. J. *Biochem. J.* **1995**, *306*, 337−344. (c) Knight, C. G.; Morton, L. F.; Onley, D. J.; Peachey, A. R.; Ichinohe, T.; Okuma, M.; Farndale, R. W.; Barnes, M. J. *Cardiovasc. Res.* **1999**, *41*, 450−457.
(7) (a) Koide, T.; Homma, D. L.; Asada, S.; Kitagawa, K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5230−5233. (b) Kotch, F.; Raines, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3028−3033.
(8) Prockop, D. J.; Fertala, A. *J. Biol. Chem.* **1998**, *273*, 15598−15604.
(9) (a) Hunter, C. A.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1990**, *112*, 5525−5534. (b) Gdaniec M.; Jankowski, W.; Milewska, M. J.; Poloński, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 3903−3906 (also, ref 9 and 10 cited therein). (c) Lozman, O. R.; Bushby, R. J.; Vinter, J. G. *J. Chem. Soc., Perkin Trans.* **2001**, *2*, 1446−1453.
(10) For details, see the Supporting Information.
(11) If the aromatic rings were placed in an edge-to-face orientation, the reminimized assembly reverted to the face-to-face orientation.
(12) Peptide **2b** did not induce platelet aggregation under various conditions.
(13) (a) Hoekstra, W. J.; et al. *J. Med. Chem.* **1999**, *42*, 5254−5265. (b) The integrin GPIIb/IIIa, which is activated at the final stage of platelet aggregation, responds to various agonists, including collagen.

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