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# Self-complementary peptides for the formation of collagen-like triple helical supramolecules

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**Abstract**—Collagen is acknowledged as one of the most prominent biomaterials on account of its high biocompatibility and biostability. The development of artificial collagens to replace the animal-derived collagens presents a challenge in the formation of safer and highly functionalized biomaterials. Here, a novel peptide-based system for obtaining collagen-like supramolecules via a spontaneous self-assembling process is described. The designed collagen-like peptides are self-complementary trimers in which each of the 24-mer peptide strands is tethered by two cystine knots forming a staggered arrangement. Their self-assembling ability in aqueous solution was analyzed by circular dichroism, ultrafiltration, and laser diffraction particle size estimation. The obtained results indicate that the staggered trimers form large supramolecular architectures through intermolecular triple helix-formation.
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Collagen is arguably the most widely used biomaterial in cosmetic surgery, tissue engineering, and drug delivery systems to date.[1,2] Animal-derived collagens purified from the tissues of cows and pigs have been generally used for such purposes. However, the use of these animal-derived collagens in human puts the recipient at risk to prion infection or even gelatin-related allergies, despite the collagen exhibiting low immunogenicity. Consequently, the production of recombinant human-type collagens and artificial collagen surrogates is of considerable interest. Recently, self-assembling peptide systems to obtain supramolecules have been extensively studied as potential methods for the formation of biomaterials.[3] In these studies, intermolecular folding to form either β-sheets[4,5] or α-helices[6] is often utilized to assemble the peptide building blocks. Although collagen-like supramolecules are expected to be highly potent biomaterials, only a few such systems have been reported to date. One such example includes an amphiphile comprising a collagen-like peptide head group and a tail group possessing either one or two alkyl chains.[7] These collagen-like peptide amphiphiles are known to self-associate through the hydrophobic interactions between their alkyl chains to form aggregated materials. A second collagen-like supramolecule comprises a collagen-triblock peptide based on the molecular structure of $(Glu)_5$-$(Gly\text{-}X\text{-}Hyp\text{-}Gly\text{-}Pro\text{-}Hyp)_6$-$(Glu)_5$, (Hyp, 4(*R*)-hydroxyproline), which forms triple helical aggregates with the aid of the Glu-repeats at either end.[8] Here, we describe a novel system for creating collagen-like supramolecules with elongated triple helical structures, based on the intrinsic triple helix-forming property of synthetic peptides with the collagenous Gly-X-Y-repeat sequences.

It is well known that collagen-like $(Gly\text{-}X\text{-}Y)_n$ peptides have an inherent propensity to fold into triple helical structures.[9,10] As occupation of X and Y positions by imino acids enhances the stability of the triple helical structure, $(Gly\text{-}Pro\text{-}Pro)_n$ is used as the simplest model of collagen triple helix. $(Gly\text{-}Pro\text{-}Hyp)_n$, the most abundant triplet repeats in native collagens, forms more stable triple helix than corresponding non-hydroxylated counterparts. The collagen triple helix is a right-handed supercoil consisting of three parallel, left-handed polyproline-II-like helices, staggered relative to each other by a single residue. The Gly residues positioned every third residue within the helical core are essential for maintaining the overall structure of the triple helix. The cooling of a typical collagen-model peptide solution causes the peptide molecules to trimerize, forming perfectly aligned (and hence) highly stable triple helices. In this manner, the lengths of the triple helices are defined by the lengths of the peptide chains, where no elongation of the triple helix is expected (Fig. 1a). It is

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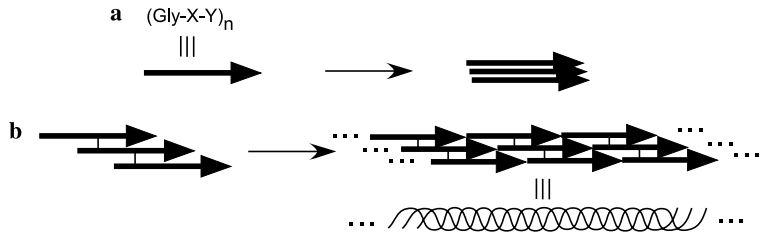



**Figure 1.** Concept for the self-assembly of collagen-like peptides. (a) Classical collagen-model peptides self-assemble to form triple helical trimers. (b) Supramolecule-formation by the intermolecular folding of self-complementary collagen-like peptides.

expected that by forcing the molecules to fold intermolecularly with regular staggering, a collagen-like supramolecule would form elongated triple helices (Fig. 1b). In order to realize this concept, we have designed two heterotrimeric peptides (**1** and **2**), which are disulfide-linked trimers of 24-mer peptides comprising similar sequences of repeat (Gly-Pro-Hyp) units (Fig. 2). The adjacent peptide strands are staggered by either 12-residues (in trimer **1**) or by 13-residues (in trimer **2**). The introduction of these staggers prevents intramolecular triple helix formation and creates self-complementary cohesive ends. Disulfide bridges between Cys residues incorporated in the X and Y positions were chosen to link the peptide strands, since such cystine-knots were reported to be structurally compatible with the collagen triple helix.[11]

Single chain precursors of the trimers (**1** and **2**) were synthesized using the standard Fmoc-based solid-phase protocol on Trt(2-Cl)-resins (Watanabe Chemical Co.). *tert*-Butyl and Trt groups were used as side-chain protecting groups for Hyp and Cys residues, respectively, while the N-terminal Cys side chains in the biscysteinyl chains were protected with acetamidomethyl (Acm) groups. After deprotection/cleavage with a trifluoroacetic acid (TFA)-scavenger system,[12] the main products were purified by reversed-phase HPLC (RP-HPLC). Heterologous trimerization was then achieved by stepwise disulfide bond-forming reactions as described in our previous paper.[13] Briefly, the [Cys(Acm),Cys(SH)]-chain was treated with dithiodipyridine (PySSPy) to activate the free thiol group (Scheme 1, step i). The pyridylthiolated peptide was then heterodimerized (step ii), and the *S*-Acm moiety was converted to an *S*-3-nitro-2-pyridinesulfenyl (Npys) group (step iii). Finally, the *S*-Npys-peptide was mixed with a second Cys(SH)-peptide to form the heterotrimer (step iv). In each step, the main products were purified by RP-HPLC and characterized by electron spray ionization mass spectrometry (ESI-MS). RP-HPLC profiles of the final products are shown in Figure 3. They are characterized as the desired products by ESI-MS: **1** *m*/*z* = 6457.2 [M]; **2** *m*/*z* = 6457.0 [M], calcd: 6456.2.

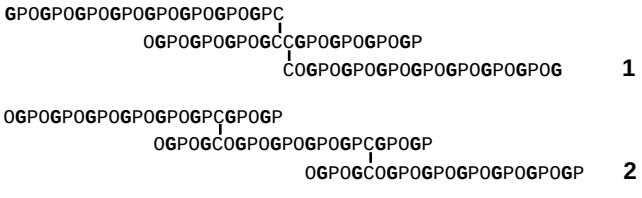
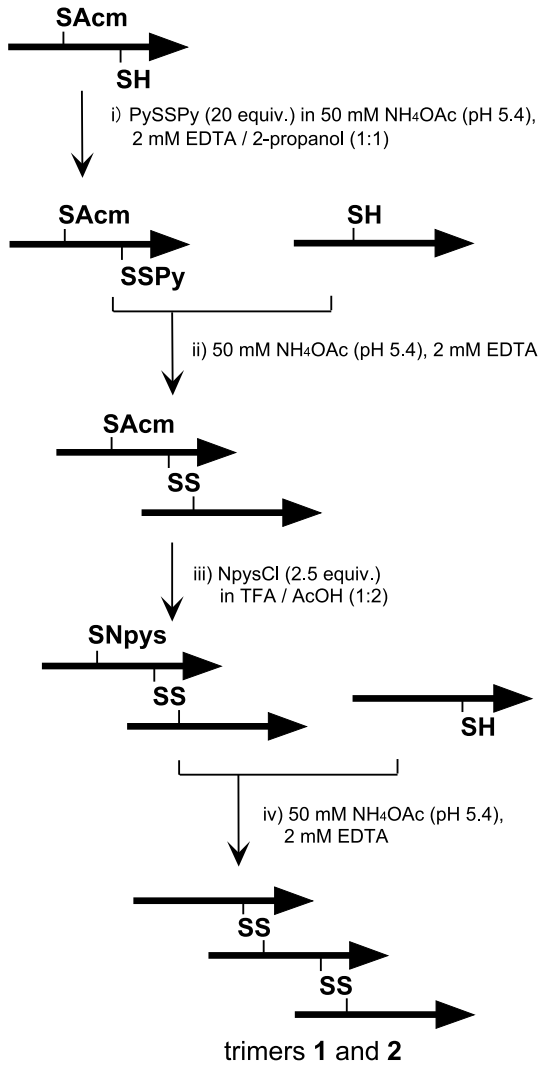



**Figure 2.** Structure of self-complementary collagen-like peptides synthesized in this study. 'O' denotes a 4-hydroxyproline residue.




**Scheme 1.** Synthesis of trimers **1** and **2**.

The lyophilized powders of trimers **1** and **2** were dissolved in water at a concentration of 10 mg/mL and kept at 4 °C for more than 3 days. In this condition, the solutions are clear and no visible precipitates were observed to form. The conformational states of trimers **1** and **2** in solution were analyzed by circular dichroism (CD) spectrometry and compared with that of the classical collagen-model peptide, $(Pro\text{-}Hyp\text{-}Gly)_8$. As shown in

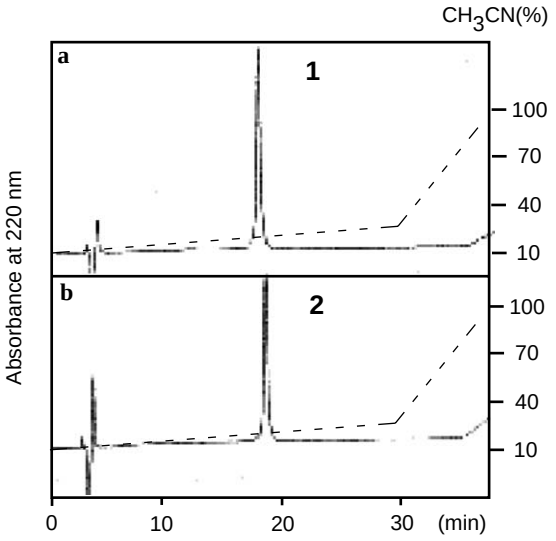



**Figure 3.** RP-HPLC profiles of the synthetic peptide trimers: (a) **1** and (b) **2**. HPLC was conducted using a Cosmosil $5C_{18}$-AR II (4.6 id × 250 mm) column with linear gradients of $CH_3CN$ in water both containing 0.05% TFA at 42 °C.

Figure 4a, all three peptides showed positive CD signals around 225 nm, indicating the formation of a collagen-like triple helix. The $R_{pn}$ values denote the ratio of positive peak intensity over negative peak intensity, and are used as an index to represent the triple helical content.[14] The $R_{pn}$ values for **1**, **2**, and (Pro-Hyp-Gly)$_8$ were estimated to be 0.12, 0.13, and 0.13, respectively, which are comparable with that of native collagen I (0.13).[14] The high triple helical contents observed in **1** and **2** strongly suggest the existence of well-organized triple helices formed between the trimer units, because intramolecular triple helix formation is very unlikely to occur. The $[\theta]_{225}$ values of the peptides were monitored with increasing temperature (Fig. 4b). The thermal melting profiles of the triple helices formed by **1** and **2** were found to differ. The triple helix formed by **2** showed a co-operative melting similar to that of the (Pro-Hyp-Gly)$_8$ triple helix, although its stability was lower than that of (Pro-Hyp-Gly)$_8$. On the other hand, the $[\theta]_{225}$ value for **1** decreased gradually with increasing temperature, suggesting that the triple helical components in solution **2** have higher homogeneity, presumably in the size of supramolecules formed.

The size of supramolecules expected to form from trimer **1** and **2** solutions was examined by measuring their permeability with respect to membranes with a 0.2 μm pore-size or a molecular weight cut-off value of 100 kDa (Fig. 5a). Native collagen I and (POG)$_8$ were used as controls. All of the solutes were found to pass through the 0.2 μm pores, while only very minor frac-

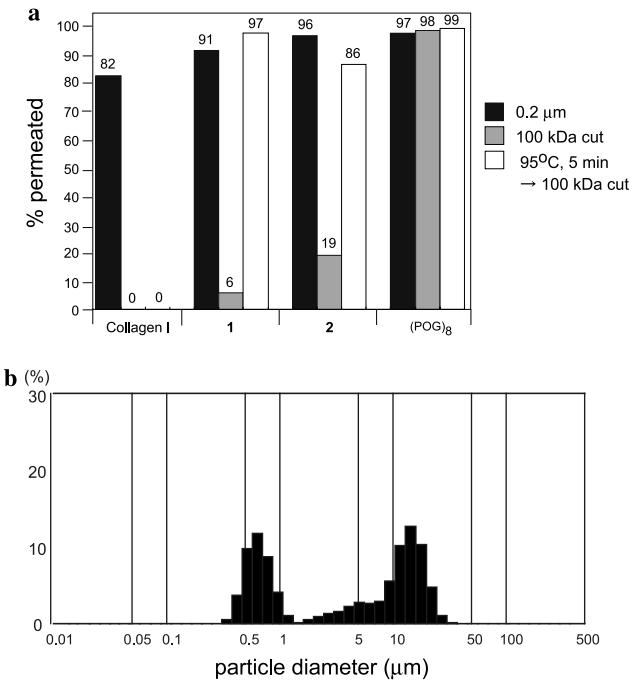



**Figure 5.** Size analysis of the collagen-like supramolecules. (a) Permeability of collagen I (Cellmatrix type I-C, Nitta Gelatin Inc., 0.3 mg/mL), trimers **1**, **2** (0.5 mg/mL), and (POG)$_8$-amide (0.5 mg/mL) to 0.2 μm-pore-size filters (Millex-LG, Millipore) and 100 kDa-molecular weight cut-off filters (Microcon YM-100, Millipore). The amount of peptides remaining in the filtrates was determined from the HPLC peak area. Collagen I was quantified by the densitometric analysis of a Coomassie blue-stained SDS–polyacrylamide gel. (b) Typical size distributions of the supramolecules formed by trimer **2**, recorded at 4 °C on a SALD-7000 Laser Diffraction Particle Size Analyzer (Shimadzu Corp.). The concentration of **2** is 0.83 mg/mL.

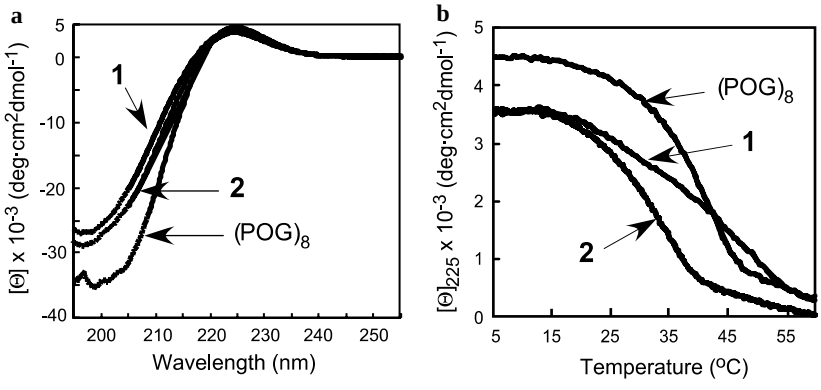



**Figure 4.** (a) CD spectra of trimers **1**, **2**, and (POG)$_8$ recorded at 4 °C. (b) Thermal equilibrium curves for the peptides monitored by CD spectroscopy at 225 nm. The temperature was increased at a rate of 0.3 °C/min.

tions of trimers **1**, **2**, and collagen I were detected in the filtrates of the 100 kDa-cut-off membranes. When the same solutions were heat-denatured at 95 °C for 5 min prior to filtration, permeability of **1** and **2** through the 100 kDa-cut-off membranes was found to be almost quantitative. The size of the supramolecules was further estimated using a laser diffraction particle size analyzer (SALD7000, Shimadzu Co.). In the trimer **2** solution, at least two prominent populations of supramolecules, giving particle sizes of approximately 0.6 and 14 μm in diameter, were detected[15] (Fig. 5b). These results, together with those obtained by CD analysis, indicate that trimers **1** and **2** form large supramolecules through intermolecular triple helix formation.

As demonstrated here, collagen-like supramolecules have been created by the self-assembly of certain trimeric peptides with purposely designed cohesive ends. The as-formed supramolecules are constructed solely from collagen-like sequences and mimic the unique tertiary structure of native collagen. At this stage, it is not clear whether the triple helices are independently dispersed in aqueous solution or form higher-order bundles like native collagen in physiological conditions. Further morphological analysis using electron microscopy or scanning probe microscopy will be able to clarify this. The use of synthetic peptides as building blocks will allow further specific functionalization of the supramolecules by incorporating functional sequences found in native collagen (integrin binding-sequences[16,17] etc). Although the thermal stabilities of these triple-helical supramolecular prototypes are still below the requirement for biological applications, the concept described here offers a novel strategy toward the development of artificial collagens.

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