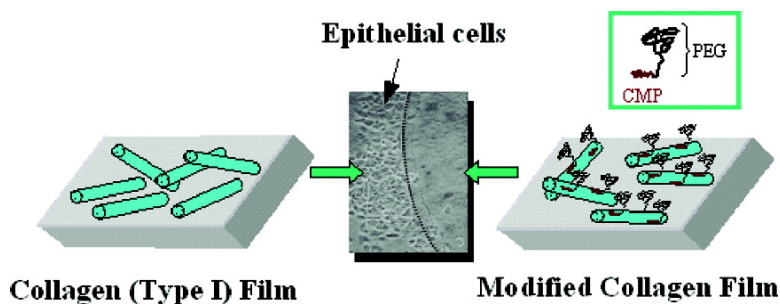


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Facile Modification of Collagen Directed by Collagen Mimetic Peptides

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Collagen is used in a variety of medical applications ranging from hemostatic materials and biocompatible coatings to drug delivery and tissue engineering. Recent widespread interest in the development of engineered tissue and organ replacement therapies has prompted demand for new approaches to immobilize exogenous components to natural collagen.^{1a–d} Chemical coupling of synthetic moieties to amino acid side chains (e.g., Lys or Glu) has been commonly practiced for such purposes; however, such coupling reactions are difficult to control on large proteins and generally are not conducive to modifying integrated collagen scaffolds that contain live cells and tissues.^{2a–c}

As an alternative to the conventional “covalent” modification methods, we have developed a novel “physical” modification technique that is based on collagen’s native ability to associate into a triple-helical molecular architecture. Here, we present a finding that collagen mimetic peptides (CMPs) of sequence $-(\text{Pro-Hyp-Gly})_x-$ exhibit strong affinity to both native and gelatinized type I collagen under controlled thermal conditions. We also show that the cell adhesion characteristics of collagen can be readily altered by applying a poly(ethylene glycol)–CMP conjugate to a prefabricated collagen film.

With more than 20 different types known to date, the collagens are among the most diverse and abundant proteins in mammals.^{3a} All collagen types share, at least in part, a common molecular architecture, a unique right-handed triple helix which is rarely found in other proteins.^{3b} Collagen mimetic peptides (CMPs) are peptides, typically of less than 30 amino acids, composed of multimers of known helicogenic peptide trimers. These CMPs have been instrumental in determining the structure and stability of natural collagens. The CMPs based on ProProGly and ProHypGly trimers are best characterized to date, and their collagen-like triple-helical structure and reversible melting behaviors are documented in the literature.^{4a–d}

We hypothesized that the propensity of CMPs to form collagen-like triple helices may enable it to bind to partially denatured collagen by associating with disentangled domains of the collagen molecules. Since CMPs can be easily conjugated to other bioactive components, such as cell adhesion peptides and growth factors, the CMP–collagen interaction may provide a convenient pathway to physically immobilize additional functionalities to collagen and gelatin.

To test this hypothesis, we prepared CMP and 5-carboxy fluorescein (5CF)-labeled CMP derivatives (Table 1, compounds **1** and **2**, respectively) and investigated their adhesion to partially denatured collagen films. In addition, peptide **3** was synthesized, which has a molecular weight and an amino acid composition similar to those of **2**, but the Pro, Hyp, and Gly sequence was scrambled, rendering it nonhelicogenic. Three consecutive glycines

Table 1. Melting Transition Temperatures of Collagen Mimetic Peptide Derivatives Determined by Circular Dichroism Spectroscopy^a

compound	sequence	T_m (°C)
1	$-(\text{ProHypGly})_{10}-$	69
2	$5\text{CF-Gly}_3-(\text{ProHypGly})_{10}-$	75
3^b	$5\text{CF-Gly}_3\text{-randomPro}_{10}\text{Hyp}_{10}\text{Gly}_{10}-$	
4	$5\text{CF-Gly}_3-(\text{ProHypGly})_6-$	25
5^c	$\text{mPEG}_{2000}\text{-Gly}_3-(\text{ProHypGly})_7-$	29

^a Measured in 57.5 μM acetic acid solution. ^b 5CF-GGGGPPP^HPP^HGGGG PP^HPP^HGP^HGP^HGP^HGP^HPP^HGGGGPP^HPP, (P^H:Hyp). ^c mPEG₂₀₀₀, CH₃O–(CH₂–CH₂–O)_n–OH, 2250 Da.

were inserted as a spacer between the fluorescence tag and the CMP. Attaching 5CF and three glycines to **1** elevated its melting temperature from 69 to 75 °C (Table 1). This is due, in part, to the hydrophobic nature of the fluorescence tag. Attachment of a hydrophobic fatty acid to the CMP was previously shown to stabilize the triple helix, elevating its melting temperature.⁵ As expected, no melting behavior was observed for compound **3**.

The binding of CMP to natural collagen (acid soluble, bovine type I) or denatured collagen (gelatin) was demonstrated by treating collagen films with solutions of the fluorescently labeled CMP, rinsing, and measuring the fluorescence intensity of the exposed film. To the collagen-coated cell culture wells (at room temperature) was added a solution of **2** which was pre-equilibrated at either 25 or 80 °C. After 3 h incubation at room temperature, the collagen films were washed with buffer solution and observed by a fluorescence microscope. As control samples, 5CF, fluorescein isothiocyanate-dextran (FITC-Dextran), and **3** were used to treat collagen films under identical experimental conditions. All control samples exhibited negligible affinity toward collagen film, evidenced by low fluorescence intensity at both experimental conditions (Figure 1A, group a and b). Collagen film treated with **2** at 25 °C also showed negligible fluorescence. In contrast, the collagen film treated with **2** that was pre-equilibrated at 80 °C, the temperature above **2**’s melting transition temperature (75 °C), exhibited strong fluorescence. Similar results were obtained when gelatin films were used as a substrate (Figure 1A, group c). In addition, the helical content of CMP-treated collagen film was 3.5 times higher than that of the film treated with a blank solution (see Supporting Information). The results suggest that **2** tightly attaches to partially denatured collagen when it is introduced as a single strand, and that its ability to assemble into a triple helix is essential for the attachment.

To understand the effect of collagen film denaturation in the CMP binding process, we synthesized a shorter CMP derivative, **4**, with a melting temperature (25 °C) well below that of the collagen film. Little binding was observed when a solution of **4** at 10 °C was used to treat the collagen film (Figure 1B). However, treatment with the same solution pre-equilibrated at 30 °C, the temperature above **4**’s melting temperature but below the denatur-

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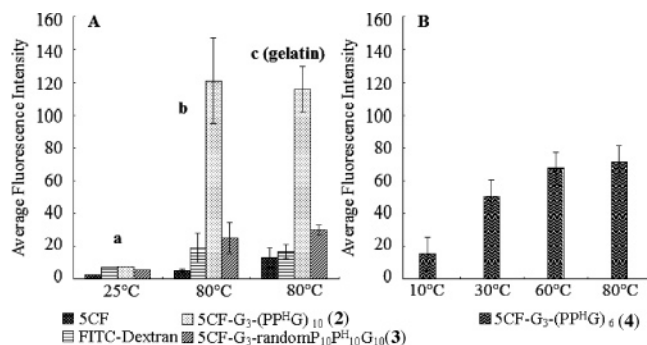


Figure 1. Fluorescence intensities of collagen films (type I, bovine) treated with 5CF-labeled CMPs and other control samples. The x-axis represents the temperature at which fluorescence solutions were equilibrated prior to addition to the collagen film (see Supporting Information). (A) Binding of **2** and control samples to collagen (group a and b) and gelatin (group c) films. The gelatin film was prepared by subjecting the collagen film to heat (80 °C) for 30 min. (B) Binding of **4** to collagen films.

ation temperature of collagen film (37 °C), induced a more than 3-fold increase in CMP attachment compared to that of the 10 °C solution. In addition, the modified collagen fiber retained its native banding texture when investigated by transmission electron microscopy (see Supporting Information). Hotter solutions (60 and 80 °C), which denature the collagen film during the treatment, produced collagen films with slightly higher CMP content (approximately 20% increase from that of the 30 °C solution). The results indicate that the CMP melting into monomers, but not collagen denaturation, is necessary and sufficient to support CMP immobilization.

We speculate that the observed adhesion arises from a strand exchange reaction and triple-helix association between CMP and the collagen. It is interesting to note that a number of researchers have proposed the existence of thermally labile domains within the type I collagen sequence which may serve as potential sites for the presumed strand exchange reaction.⁶ Within the collagen family exists a class of collagen known as Fibril Associated Collagens with Interrupted Triple-Helices (FACITs)^{7a-c} that do not form fibrous structure by themselves but are always found as individual collagen molecules decorating the surface of collagen fibers. Our current findings do not indicate if CMP is binding to the thermally labile domains, or if the binding event mimics that of the FACIT proteins. We are currently addressing these questions by calorimetric analysis and transmission electron microscopy.

To evaluate the potential of the new modification technique in tissue engineering, we prepared a poly(ethylene glycol)₂₀₀₀-CMP conjugate polymer^{8a,b} (Table 1, **5**) that is designed to reduce the adhesiveness of collagen to cells.

The melting temperature of **5** was determined to be 29 °C, which is 7 °C lower than the melting temperature of (ProHypGly)₇ (Table 1).⁹ Here, the hydrophilic and bulky PEG group seems to destabilize the triple helix in water. A solution containing **5** at 45 °C was added to the collagen-coated culture plate (prepared as above), and human fibroblasts or breast epithelial cells were seeded and incubated for 3 days at 37 °C. Homogeneous distribution of fibroblasts is seen on the collagen film that was treated with mPEG₂₀₀₀ (control sample, Figure 2A). In contrast, areas of collagen films treated with **5** are almost devoid of fibroblasts (Figure 2B) and epithelial cells (Figure 2C). This experiment demonstrates that the adhesive properties of prefabricated collagen film can be readily modified by the simple action of delivering CMP conjugate solutions to the target area.

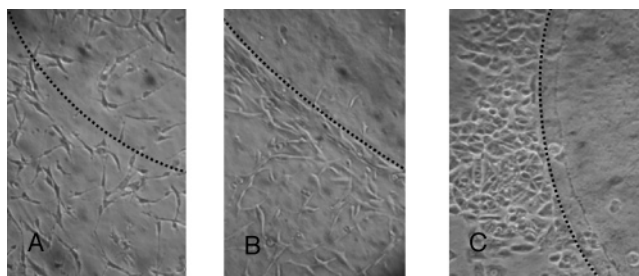


Figure 2. Optical micrographs of human fibroblasts (A and B) and breast epithelial cells (C) cultured on collagen films that were pretreated with mPEG₂₀₀₀ (A) or **5** (B and C). Areas of the picture to the right of dotted lines were treated with mPEG₂₀₀₀ or **5**.

The ability to control the organization of cells in collagen matrix may provide a new pathway for engineered tissues.¹⁰ Furthermore, the affinity between the CMP and collagen could be used to immobilize therapeutic drugs to collagens in the living tissues and to biomaterials that incorporate natural collagens. We are currently focusing on expanding the modification technique to 3D collagen scaffolds.

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Supporting Information Available: Materials and methods, CD melting curves, helical content study, and TEM of modified collagen fibers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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