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Spatio-Temporal Modification of Collagen Scaffolds Mediated by Triple Helical Propensity

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Abstract

Functionalized collagen that incorporates exogenous compounds may offer new and improved biomaterials applications, especially in drug-delivery, multifunctional implants, and tissue engineering. To that end, we developed a specific and reversible collagen modification technique utilizing associative chain interactions between synthetic collagen mimetic peptide (CMP) [$(ProHypGly)_x$; Hyp = hydroxyproline] and type I collagen. Here we show temperature-dependent collagen binding and subsequent release of a series of CMPs with varying chain lengths indicating a triple helical propensity driven binding mechanism. The binding took place when melted, single-strand CMPs were allowed to fold while in contact with reconstituted type I collagens. The binding affinity is highly specific to collagen as labeled CMP bound to nanometer scale periodic positions on type I collagen fibers and could be used to selectively image collagens in ex vivo human liver tissue. When heated to physiological temperature, bound CMPs discharged from the collagen at a sustained rate that correlated with CMP's triple helical propensity, suggesting that sustainability is mediated by dynamic collagen–CMP interactions. We also report on the spatially defined modification of collagen film with linear and multi-arm poly(ethylene glycol)-CMP conjugates; at 37 °C, these PEG-CMP conjugates exhibited temporary cell repelling activity lasting up to 9 days. These results demonstrate new opportunities for targeting pathologic collagens for diagnostic or therapeutic applications and for fabricating multifunctional collagen coatings and scaffolds that can temporally and spatially control the behavior of cells associated with the collagen matrices.

Introduction

Collagens, the most abundant protein in mammals, provide a structural framework during tissue development and repair. They are known to induce proliferation and differentiation of

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Supporting Information Available. Figures S1–S6, including fluorescence optical micrographs of human liver carcinoma slices stained with anti-CD31-(*R*)-PE antibody and varying concentrations CMP-6, cumulative release profiles of ^{F}CMP-Xs from collagen gels, optical micrographs of PEG-CMPX and MPEG-CMP8 treated collagen films seeded with MCF-7, structure of MPEG-CMP8, GPC traces of MPEG-CMP8 and ^{F}MPEG-CMP8 conjugates, a picture of self-supporting ^{F}MPEG-CMP8 hydrogel and CD traces of all CMP derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

cells through direct binding interactions and by serving as reservoirs for growth factors and signaling molecules. Due to their abundance, collagens (especially type I and II collagen) can be readily isolated from animal tissues, stored in a solid or solution form and used as biocompatible coatings or soft-tissue substitutes that can protect and support damaged tissues (e.g., corneal shields, anti-infectious catheters, tissue sealants, hemostatic sponges, and topical wound dressing products).[1] Lately, collagen films and gels have been widely used as tissue scaffolds for skin replacement,[2] bone substitute,[3] and artificial blood vessels and valves.[4] Despite many attributes ideal for tissue formation (e.g., high porosity, low immunogenicity, and bioresorbability), purified collagens lack the full complement of ingredients necessary for the development of specialized tissue, necessitating that culture media be supplemented with tissue specific growth factors. Implanted collagen is also known to cause infection, calcification, and formation of pathogenic scar tissues.[1] Therefore, biomaterial scientists have had a longstanding interest in modifying natural collagen to circumvent these limitations while preserving the benefits of the natural extracellular matrix (ECM) protein.[5–7]

Past approaches to improve the performance of commercially available collagens for use in drug delivery and tissue engineering have met with limited success. Due to the loose network structure and hydrophilic nature of the collagen fibers, bioactive compounds added to collagen passively discharge from the collagen matrix at a rapid and uncontrolled rate. For example, approximately 75% of bone morphogenic growth factor (BMP) is released from a collagen sponge within 3 h after charging despite BMP's large size.[8] Covalent chemical conjugation reactions have produced collagens with antimicrobial activity or higher affinity to bioactive compounds; however, the activity of the immobilized components and their efficacy in therapeutic setting remains to be elucidated.[9,10] In general, chemical reactions are limited by the low reactivity of the protein and can compromise the biochemical features of natural collagens. In addition, the potential toxicity and nonspecific reactivity of chemical reactions are not conducive to modifying collagen scaffolds that contain cells and other bioactive components.

The triple-helical structure of collagen has fascinated scientists for its unique multiplex architecture and its essential role in the formation of both functional collagen molecules and higher order fiber assembly. Collagens are composed of three separate protein strands that are rich in proline and hydroxyproline (Hyp), which confer rigidity to the backbone conformation.[11–13] Periodic glycine repeats at every third residue allow the three collagen strands to pack closely and form a stable triple helix through interchain hydrogen bonds around a common axis similar to a DNA double helix.[14] The triple helix can be found in all 28 collagen types reported to date[15] as well as a few noncollagen proteins such as complement protein and mannose binding proteins.[11,16] Synthetic collagen model systems, also known as collagen mimetic peptides (CMPs), have been useful in elucidating collagen structure and factors responsible for the stabilization of the triple helix.[9,17–19] CMPs form a triple helical structure almost identical to that of natural collagens; however, unlike collagen molecules, CMPs are small (<3 KDa) and, therefore, exhibit reversible melting behaviors.[11,13,17] CMPs based on ProProGly or ProHypGly trimers have been widely studied and their melting temperatures (T_m) vary from 21 to 75 °C, depending on the molecular weight and amino acid composition.[9,11–13,18–20]

Considering the rare occurrence of triple helical protein structure in nature outside of collagen, we thought that the associative interactions between collagen strands in forming the triple helix can be explored as a unique physical conjugation tool for attaching exogenous molecules to natural collagens. Physical interactions, such as hydrogen bonding, hydrophobic, and ionic interactions and biological affinity interactions, provide a much more convenient and natural way of conjugating agents to proteins and other

macromolecules as opposed to covalent chemical attachment.[21] For example, biotin–avidin interactions, metal-mediated histidine–NTA interactions, and antigen–antibody binding are all effective protein conjugation tools in biology research. Recent studies on collagens have shown that even at physiological conditions, type I collagen is thermally unstable.[22] In addition, sequence analysis has revealed blocks of less-structured domains[23] within the type I collagen molecules. Our working hypothesis was that these partially unraveled domains within the collagen molecules may be able to associate with other small peptides of strong triple helical propensity such as CMPs.

In an effort to develop a new method for noncovalent collagen scaffold modification, we investigated the folding behavior of CMPs in contact with natural collagens. This led to the discovery of CMP's structure-dependent binding affinity to type I collagen which has been applied to the fabrication of collagen substrates that repel cell attachment[19] and a poly(ethyleneglycol)–collagen hybrid scaffold ideal for chondrocyte and mesenchymal stem cell encapsulation.[24] In this article, we present temperature-dependent collagen binding/release studies and nanoparticle localization experiments of a series of CMPs that provide new insights into the mechanism of CMP–collagen interactions. We also provide histological analyses of human liver tissue using labeled CMPs that highlight CMP's remarkable binding specificity to human collagens. Finally, we report on the synthesis of both linear and multiarm poly(ethyleneglycol)-CMP conjugates and discuss their collagen binding/release characteristics as well as the ability to repel attachment and advancement of mammalian cells on collagen films. We believe that such CMP-collagen conjugates can be used to control the spatial and temporal distribution of cells in collagen-based tissue scaffolds and thus presents an innovative approach for advancing tissue engineering that improves upon both the specificity and the practical utility of current conjugation methods.

Experimental Section

Materials

Fmoc-amino acids, 2-(1*H*-benzo-triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Advanced ChemTech (Louisville, KY). Rink amide resin was purchased from NovaBiochem (San Diego, CA). TentaGel PAP resin was purchased from Rapp-Polymere (Tübingen, Germany). Acid soluble, type I bovine collagen was purchased from Sigma (St. Louis, MO). Fluorescein-PEG_{2000}-NHS and methoxy-PEG_{2000}-NHS were purchased from Nektar Therapeutics (Huntsville, AL), while 4-arm PEG-NHS (PEG-tetraglutaryl NHS, average Mw: 11000) was purchased from NOF Corporation (Tokyo, Japan). ^{F}CMP-6 was purchased from Genscript Corporation (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich and used without further purification. MCF-7 (human breast epithelial cell line) was obtained from ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) purchased from Invitrogen Corporation (Carlsbad, CA), supplemented with 10% FBS/100 units/mL penicillin and streptomycin.

Synthesis and Purification of Collagen Mimetic Peptide (CMP) Derivatives

The peptides were synthesized by condensation of the corresponding Fmoc-amino acids (Fmoc-Gly-OH, Fmoc-Hyp-OH, Fmoc-Pro-OH, Fmoc-Cys(Trt)-OH and Fmoc-Lys(Mmt)-OH), and 5-carboxyfluorescein (5CF) on a solid support (Fmoc-Gly-Wang resin, Rink amide resin, or TentaGel PAP resin) as reported previously.[12] The CMPs were cleaved from resins by treatment with water/triisopropylsilane/ trifluoroacetic acid (2.5:2.5:95%) for 3 h and the PEG-CMP8 and PEG-CMP10 were cleaved from TentalGel resins by treatment with trimethylbromosilane/thioanisole/trifluoroacetic acid (1:5:95%) for at least 4 h. The crude CMP derivatives were precipitated with cold ether and dried in vacuum. Reverse-phase

HPLC purification was performed on a Varian Polaris 210 series liquid chromatograph with a semipreparative Vydac C-18 reversed-phase column at a flow rate of 4 mL/min. Gel permeation chromatography purification for PEG-CMPXs was performed on a Shodex protein KW-802.5 column at a flow rate of 0.3 mL/min, and the purification was performed slightly above PEG-CMPX's T_m. The purified peptides were analyzed by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied Biosystems, Foster City, CA). ^{F}CMP-7 $[M + Na]^+$ = 2434.3 Da (calcd = 2434.5 Da); ^{F}CMP-10 $[M + H]^+$ = 3220.2 Da (calcd = 3220.3 Da); ^{F}CMP-R10 $[M + H]^+$ = 3221.7 Da (calcd = 3220.3 Da); ^{F}PEG-CMP7 $[M + H]^+$ = 4684.3 Da (calcd = 4686.5 Da); ^{F}PEG-CMP8 $[M + H]^+$ = 7951.8 Da (calcd = 7956.9 Da); ^{F}PEG-CMP10 $[M + H]^+$ = 8486.2 Da (calcd = 8491.5 Da); PEG-CMP7 $[M + Na + K]^+$ = 4305.1 Da (calcd = 4304.2 Da); PEG-CMP8 $[M + H]^+$ = 7425.2 Da (calcd = 7425.2 Da); PEG-CMP10 $[M + H]^+$ = 7961.3 Da (calcd = 7944.0 Da); CMP-8 $[M + H]^+$ = 2325.3 Da (calcd = 2328.4 Da); ^{F}CMP-8 $[M + H]^+$ = 2830.6 Da (calcd = 2832.0 Da), CysCMP-7 $[M + H]^+$ = 1991.1 Da (calcd = 1992.1 Da); Cys-CMP-R7 $[M + H]^+$ = 1991.2 Da (calcd = 1992.1 Da).

$^{(F)}$CMP-Xs Binding Affinity to Collagen Films

To each well of a 48-well tissue culture plate, 100 μL of 5 mg/mL collagen (acid-soluble, type I, bovine) solution in 0.5 M acetic acid was added and air-dried. This procedure was repeated four times to make stable collagen films on the culture plate surfaces. The films were washed with distilled water, neutralized with 0.01 M potassium phosphate buffer (pH 7.4), and washed thoroughly with distilled water. For the binding affinity study of ^{F}CMP-6 at a 30 °C preincubation temperature, 1 μL of 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM of ^{F}CMP-6 was pre-equilibrated at 30 °C and added to the dry collagen-coated wells sitting at room temperature. This procedure was repeated 10 times. After 3 h of incubation at room temperature, the film was allowed to dry, and unbound materials were removed by washing with 4 °C, 0.01 M potassium phosphate buffer (pH 7.4) and distilled water. The total amount of washing volume was 10 mL. The 10 °C experiment was incubated at 10 °C for 3 h and similarly washed with PBS buffer (pH 7.4) at 4 °C. The concentrations of ^{F}CMP-Xs of the wash solutions were determined by measuring absorbance at 493 nm using a UV–vis spectrophotometer. Average values of four independent experiments are reported in Figure 1. For initial dissociation index (IDI) determination, ^{F}CMP-X ranging from 1 to 500 nM was added to type I collagen-coated tissue culture plates under their respective maximum binding condition (marked M in Figure 1). The level of binding was determined by measuring the UV–vis absorbance of wash solutions and the IDI was calculated using a modified Scatchard analysis.[25]

Regeneration of Type I Collagen Fibers and TEM Sample Preparation

Collagen fibers were regenerated according to the literature.[26] Fiber formation was achieved by combining acid soluble collagen (type I mouse tail tendon, 0.5 mL, 0.6 mg/mL) in 2 mM acetic acid solution (4 °C) with 0.5 mL of 60 mM sodium phosphate buffer (pH 7.4, 4 °C) containing 300 mM sodium chloride and 60 mM *N*-[tris(hydroxymethyl)methyl-2-amino] ethanesulfonic acid (TES). The mixture solution was incubated at 4 °C for 6 h. For TEM sample preparation, a drop (8 μL) of type I collagen fiber solution (0.2 mg/mL in PBS containing 1% BSA) was applied to a holey carbon TEM grid. Nanoparticle-CMP solution (8 μL, 3 nM in PBS containing 1% BSA) was applied to the grid and incubated for 5 min, followed by washing with deionized water. The collagen fibers were stained with uranyl acetate (1% solution).

Synthesis and Purification of $^{(F)}$MPEG-CMP8 Conjugates

For the ^{F}CMP-8 synthesis, a CMP8 with C-terminus Lys ε-protected with acid-labile 4-monomethoxytrityl (Mmt) was synthesized on a TentaGel S RAM resin. The Mmt group

was removed by treatment with a mixture of 95% DCM, 4% TIS, and 1% TFA for 5 min. The procedure was repeated 10 times, followed by DCM wash and the active ε-amine was confirmed by ninhydrin test. The resin was preswollen in DIPEA/DCM solution to which 5-carboxyfluorescein *N*-succinimidyl ester (5-fold molar excess) was added and the reaction was run in the dark for one week. The peptide was cleaved from the resin and purified using HPLC as described before.

Four-arm PEG-NHS (2.73×10^{-6} mol) in 600 μL of 50 mM aq $NaHCO_3$ (pH 8.3, 60 °C) was mixed with purified $^{(F)}$CMP-8 ($1.23 \times 10{-}5$ mol) in 400 μL of 50 mM aq $NaHCO_3$ (pH 8.3, 60 °C). The reaction was carried out at 60 °C for 2 days and terminated by the addition of 10 μL of 4 M aq hydroxylamine (pH 8.6). The crude product was dialyzed against deionized water using a SpectraPro dialysis membrane (MWCO 12000~14000) at 60 °C and lyophilized prior to use. ^{1}H NMR of MPEG-CMP8 indicated an overall functionalization of 82.8% of PEG arms, corresponding to an average of 3.3 out of 4 arms. ^{1}H NMR (D_2O): δ 3.70 ppm (1000H, PEG backbone, -CH_2CH_2O-, s) and 1.96–2.44 ppm (192H, $C_\beta H_{l,h}$ and $C_\gamma H_2$ of Pro as well as $C_\beta H_{l,h}$ of Hyp, m).[27,28] The gel permeation chromatography was used to analyze $^{(F)}$MPEG-CMP8 conjugates. For solvents, PBS (pH 7.5) with 0.2 M NaCl was used at a flow rate of 0.5 mL/min. Agilent 1200 series was equipped with three tandem columns (Agilent PL aquagel-OH 30, 40 and mixed) and an RI detector. GPC analysis was performed at 40 °C.

Thermal Melting Temperature Measurement

Circular dichroism (CD) spectra (Figure S7) were recorded on a JASCO 710 spectrometer equipped with JASCO PTC-348 WI temperature controller and Hellma cell (400 μL, 0.1 mm path length). The thermal melting curves were obtained by measuring the molar ellipticity at 225 nm with 1 °C/min heating rate. All samples (57.5 μM in pH 7.4 PBS)[12] were equilibrated at 4 °C for 24 h before the CD measurement. The concentration of samples was determined by UV–vis spectrophotometer at 214 or 493 nm.[12,29]

Staining Native Collagen in Unfixed Human Liver Carcinoma Slices with ^{F}CMP-6

Human liver carcinoma slices were obtained from Ralph Hruban (Johns Hopkins University) using IRB exemption No. 05-07-22-04e. These tissues were fresh-frozen at the time of biopsy and remained frozen until use. The tissues were never fixed in any way and were sliced to 15 μm on charged glass slides. ^{F}CMP-6 and ^{F}CMP-R10 were both heated at 37 °C for 20 min prior to addition to the slices, which were at room temperature (23 °C) throughout the staining process. The ^{F}CMP-Xs were applied as a 50 μM (or as other concentrations for dose escalation studies) solution in 100 μL of PBS buffer along with anti-CD31-(R)-PE (Becton Dickenson, Franklin Lakes, NJ) and anticollagen I~V (Abcam, Cambridge, MA). The anticollagen I~V was detected with a secondary antirabbit-Alexa Fluor immunoconjugate. The slides were washed and mounted with a glass coverslip. The slices were viewed using a Zeiss LSM 510 Meta confocal microscope using a 488 nm laser line to excite both the ^{F}CMP-Xs and the anti-CD31-(R)-PE. The secondary immunoconjugate was excited using a 610 nm laser and the emission filters were 505–530 nm (green), 565–596 nm (red), and 647–743 nm (blue). All images were acquired at 20× magnification using a 90 × 90 μm field of view. For the blocking experiment, the tissue sample was first treated with GlyGlyGly-$(ProHypGly)_6$ (100 μL, 100 μM) before similarly staining with ^{F}CMP-6 and other antibodies.

Release Study of CMP and PEG-CMP Derivatives from Collagen Matrices

Collagen films were prepared in 48-well tissue culture plate and charged with ^{F}CMP-Xs or ^{F}PEG-CMP derivatives following the maximum loading condition for each CMPs (marked M in Figure 1) as described before. After 3 h of incubation at room temperature, the

collagen film was allowed to dry and unbound materials were washed with 4 °C, 0.01 M potassium phosphate buffer solution (pH 7.4) until no more fluorescence was detected in the wash solution. The total amount of washing volume was 10 mL. The wells were filled with 500 μL of PBS buffer (pH 7.4) and the culture plate was incubated at 37 °C (5% CO_2) with PBS buffer exchange at every 48 h. The concentrations of CMP released during incubation were determined at 2 day intervals by measuring UV–vis absorbance at 493 nm. Average values of four independent experiments are shown in Figure 4 and 5.

^{F}CMP-Xs release from collagen gel was studied in a similar manner as the col0lagen film experiment. Collagen gels (50 μL, acid-soluble type I collagen, rat tail) were prepared following a protocol provided by BD Bioscience and transferred to 48-well culture plate. ^{F}CMP-X (50 μL, 2 mM) was applied to each collagen gel under the same loading conditions as those for the collagen film experiments. After a 3 h incubation at room temperature, the gels were washed with 4 °C PBS buffer and the ^{F}CMP-X release profiles at 37 °C were recorded as described before (Figure S2).

Cell Attachment Study of PEG-CMPX and MPEG-CMPX Modified Collagen Films

To test the cell repelling activity of PEG-CMPXs and MPEG-CMPXs, the collagen films were prepared in 48-well culture plate and charged with the target compound as described before. Human breast epithelial cells (MCF-7, 500 μL of 5.6×10^5 cells/mL in DMEM) were added to the culture well and incubated at room temperature for 30 min. The nonattached cells were removed by rinsing the well with growth medium and PBS buffer. The remaining cells were incubated at 37 °C (5% CO_2), and the growth medium was exchanged every other day. Cell attachment was studied with an optical microscope on the third, fifth, seventh, ninth, 11th, and 12th day (Figure S6).

Results and Discussion

To characterize CMP-collagen binding affinity, we used Fmoc-mediated solid phase peptide coupling to synthesize several fluorescent (5-carboxyfluorescein; 5CF) CMP derivatives, each with a different T_m, referred to as **^{F}CMP-6**, **-7**, and **-10** (Table 1). As a control, we prepared a peptide, **^{F}CMP-R10**, with identical amino acid content as **^{F}CMP-10** but scrambled in sequence and unable to support the formation of a triple helix. Each 5CF-labeled peptide contained a triple-glycine spacer. T_ms of the peptides in the absence of collagen were determined in PBS (Table 1). The T_ms are slightly higher but comparable to those reported for similar CMPs without 5CF. Both the T_m and circular dichroism spectroscopy values reflect the strength and stability of the CMP triple helices formed and correlate strongly with peptide lengths.[12,19,29] As expected for a molecule unable to form triple helices, no melting behavior was observed for the control **^{F}CMP-R10**.

To determine the binding affinity of CMP to collagen, ^{F}CMP-Xs in solutions preincubated at four different temperatures (10, 30, 45, and 80 °C) were applied to room temperature cell culture plates coated with stable type I collagen (bovine) film. We used temperatures that fall between and outside the three ^{F}CMP-Xs' T_ms to test the effect of CMP melting on collagen binding. For all temperatures except 10 °C, culture plates were incubated at room temperature (22~23 °C) for 3 h after CMP application and then washed extensively with 4 °C PBS until no more fluorescence was detected in the wash solution. The 10 °C experiment was incubated at 10 °C for 3 h and similarly washed with 4 °C PBS buffer. The amount of peptide remaining on the collagen film was determined by measuring the fluorescence intensity of the wash solutions (Figure 1).

CMP binding remained low at preincubation temperatures falling below that particular CMP's T_m, while preincubation temperatures above the T_m increased binding up to 9-fold.

None of the ^{F}CMP-Xs preincubated at 10 °C bound to the collagen film, most likely because this temperature is far below all three T_ms and the ^{F}CMP-Xs were in stable triple-helical homotrimers when introduced to the collagen film, providing no driving force for collagen interaction. Compared to 10 °C, binding was higher when 30 °C ^{F}CMP-Xs solutions were applied to the collagen film. **^{F}CMP-6** exhibited significantly higher binding, 2- and 4-fold higher than **^{F}CMP-7** and **^{F}CMP-10**, respectively. Because **^{F}CMP-6**'s T_m is 21 °C compared to 36 °C for **^{F}CMP-7** and 75 °C for **^{F}CMP-10**, presumably only **^{F}CMP-6** is in a melted monomeric state at 30 °C, conducive to collagen binding. At 45 °C, both **^{F}CMP-6** and **-7** exhibited similarly high levels of binding, and at 80 °C all three CMPs bound to the collagen film. In contrast, **^{F}CMP-R10** displayed negligible binding under all experimental conditions, similar to the results we obtained previously for this peptide and other control compounds such as 5CF and fluorescently labeled dextran.[19]

CMP binding correlated to a lesser extent with the degree of collagen film denaturation upon contact with hot CMP solutions. The typical denaturation temperature of type I collagen is 37 °C. Independent experiments under identical conditions indicated that 45 and 80 °C blank solutions denature our collagen film by up to 13 and 42%, respectively.[19] Our results suggest that CMP binding is enhanced when collagen film is slightly denatured but is reduced when denaturation exceeds 42%. This is evidenced by the modest increase in **^{F}CMP-6** binding from 30 to 45 °C, and the decrease in binding from 45 to 80 °C for both **^{F}CMP-6** and **-7**. Moderate heat seems to create additional reactive sites in collagen by partially denaturing the film; however, that extensive denaturation diminishes binding suggests that some level of preorganization of the collagen molecule facilitates CMP binding.

To obtain more quantitative binding affinity measurements, we determined IDI (Table 1), measured in a similar manner as dissociation constants (K_d),[25] for each ^{F}CMP-X under their respective maximum collagen binding conditions (marked M in Figure 1). In the binding experiment, the effective concentration of CMP is dramatically reduced because, during cooling, CMP can self-assemble to form trimers that have no affinity to collagen. Therefore, the IDI values we calculated are not exact K_d measurement but likely represent the upper limit of the true K_d. As expected, the **^{F}CMP-10** produced the lowest IDI value at 1.4×10^{-8} M, while the **^{F}CMP-7** and **^{F}CMP-6** were 1.9 and 2.3 times higher than ^{F}CMP-10, respectively. The scrambled sequence **^{F}CMP-R10** had a nonspecific binding IDI that was at least 2 orders of magnitude higher than triple helix forming **^{F}CMP-Xs**. The results are in agreement with our hypothesis that CMP with higher T_m and stronger triple helical propensity binds more tightly to collagens.

The discrepancy in size and structural complexity of small CMP molecules bound to large collagen make it difficult even with modern spectroscopic and diffraction techniques to determine the molecular structure of the bound peptide and the location of the binding sites. To examine the localization of CMP binding, we sought to visualize the CMP–collagen complex using transmission electron microscopy (TEM) and electron dense gold nanoparticles (NP) functionalized with Cys-CMPs. We previously reported the poly(proline)-II-like structure of single-stranded CMPs anchored to the surfaces of these gold nanoparticles, as well as the NPs' high colloidal stability in a wide range of salt and pH conditions and their binding affinities to type I bovine collagen fibers.[18] In our current study, we tested type I collagen isolated from mouse tails, which is known to form higher quality collagen fibers with more prominent banding features compared to bovine collagen previously tested.[26] Room temperature incubation of these collagen fibers with gold NPs displaying Cys-(ProHypGly)$_7$ (**CysCMP-7**, Table 1) produced intact fibers decorated with a large number of NPs similar to those observed for bovine type I collagen.[18] Surprisingly, NPs were present on some collagen fibers with a marked periodicity along the long fiber

axes (arrows, Figure 2a). Control NPs with nonhelicogenic scrambled peptide sequences, such as **CysCMP-R7** (Table 1), exhibited much lower and nonperiodic affinity to the collagen fibers (Figure 2b). Careful analysis of the collagen banding pattern (negative staining)[30] and comparison of the bands and NP positions revealed that these positions were located precisely at one interface between the overlap (white band) and gap (black band) regions facing the collagen molecule's C-terminal end. According to the quarter-staggered fiber packing theory,[30,31] this interface contains the C telopeptide and Hyp deficient domains, both of which form structurally loose triple helices.[32] Although this domain includes many charged amino acids, charge interactions by themselves did not promote NP binding because NPs with a scrambled sequence did not bind the collagen fibers.

We believe that the observed adhesion between CMP and type I collagen arises from a single-stranded CMP interacting with collagen molecule and forming a stable product secured by a triple helix assembly. The most likely targets of interaction, as suggested by the NP experiments, are areas of collagen with a loose triple helical structure. The collagen triple helix is stabilized by Hyp and Pro amino acids, and collagen domains lacking these amino acids in all three chains are believed to form a loose and unstable triple helix that "breathes heavily".[32,33] Presumably, the small size and high Pro and Hyp content of single-stranded CMP allow it to intercalate into those loose areas and form stable triple helix complexes with collagen chains. Recent work has shown that unstable CMPs can be rescued by CMPs of strong helical propensity to form stable hetero trimeric helices.[34] At this point, more work is needed to verify the precise mechanism of CMP binding, but our results indicate that CMP's binding affinity is determined by its propensity to form a triple helix and that intact type I collagen fibers present well-defined domains that are particularly receptive to CMP binding.

We next characterized the binding specificity of CMP for native collagen in unfixed human liver carcinoma slices. Human liver contains an abundance of collagen in both the basement membrane and within portal tracts, which are easily identified structures.[35] We used **^{F}CMP-6** for this study because it displayed the maximum binding affinity at 30 °C, which is well below collagen denaturation of human tissue. Fresh-frozen human liver carcinoma slices were treated with 37 °C solutions containing either **^{F}CMP-6** or the control peptide **^{F}CMP-R10**. All slides were costained with a commercial polyclonal antibody reactive to collagens type I~V and an anti-CD31-(*R*)-PE monoclonal antibody which serve as positive control and visual landmark, respectively.[36,37] Although our previous experiments only demonstrated CMP's binding affinity to type I collagen, we expected that **^{F}CMP-6** staining would colocalize with anticollagen I~V antibody because all types of collagens incorporate, at least in part, triple helical structure and some spectrum of helical stability. Confocal fluorescence microscopy (Figure 3a) confirms that **^{F}CMP-6** colocalizes with the anti-collagen I~V antibody. In contrast, the **^{F}CMP-R10** peptide showed no discernible binding (Figure 3b) and no specific fluorescence were observed for unstained tissue samples. Pretreating the tissue sample with an excess of GlyGlyGly-$(ProHypGly)_6$ blocks **^{F}CMP-6** staining by saturating the collagen's CMP binding sites (Figure 3c). This suggests the presence of well-defined binding domains which is in agreement with the NP labeling results. In addition, dose escalation of **^{F}CMP-6** in serial slices of human liver tissue showed a corresponding dose-dependent increase in fluorescence intensity of collagen-bound **^{F}CMP-6** (Figure S1). The results show that **^{F}CMP-6** is capable of recognizing and binding multiple types of native collagens in an ex vivo setting. In contrast to anticollagen antibodies,[38,39] CMPs are modifiable, low molecular weight compounds (<2 kDa) that can be conjugated to imaging or therapeutic agents. With their strong and diverse binding ability, they may prove useful for studying, diagnosing, and treating diseases accompanied by pathologic scar tissue formation or other extracellular matrix abnormalities.[35,40–46]

We predicted that when a CMP–collagen complex is heated to a temperature close to the T_m of CMP or collagen, the CMP would dissociate from the collagen and be released into surrounding environment, a process that can be explored for sustained or triggered drug delivery. We investigated this hypothesis by studying the release profiles of ^{F}CMP-Xs at physiological temperature (37 °C) from collagen films with maximum peptide loading levels (marked M in Figure 1). Figure 4 shows the cumulative release profiles over a 12-day period at 2-day intervals (buffer replaced every other day). Day 0 represents the initial ^{F}CMP-X loading levels after extensive washing with 4 °C buffer to remove unbound ^{F}CMP-Xs. **^{F}CMP-R10**, incapable of forming triple helices due to scrambled sequence, was released immediately during the washing process whereas all three structural CMPs had comparable initial loading capacities (~300 nmol/cm^2). After washing, the films were incubated at 37 °C and their CMP release profiles were recorded by measuring the UV–vis absorbance of the ^{F}CMP-X released into the solution.

^{F}CMP-6, which has the lowest triple helical stability among the three peptides tested (T_m: 21 °C), discharged from the collagen matrix within 3 days and showed little sustainability. In contrast, **^{F}CMP-7**, with higher helical stability (T_m: 36 °C), exhibited a sustained release profile with only 58% release at day 3 and 90% release at day 5. For **^{F}CMP-10** (T_m: 75 °C), two distinct populations were observed; approximately 50% of the peptides discharged in a sustained release fashion but at a significantly slower rate than **^{F}CMP-7**, while the other 50% did not dissociate even after 12 days of incubation at 37 °C.

At 37 °C, all three ^{F}CMP-X-collagen complexes are no longer stable and the peptides are released from the collagen matrix; however, the drastic differences in the release profiles despite similar initial loading at day 0 suggest that the modes of release are quite different for the three CMPs. For **^{F}CMP-6**, immediate release without any sustainability suggests that the **^{F}CMP-6**–collagen complex is completely melted at this temperature and the dissociated peptide bears no attraction to the collagen matrix. **^{F}CMP-7** is also entirely released from collagen at physiological temperature; the marked sustainability of the release profile, however, suggests that the peptide is interacting dynamically with the collagen matrix presumably by partial structure formation. We believe that thermal instability and the dynamic interaction between peptide and collagen also leads to the partial sustained release of **^{F}CMP-10**. But due to high triple helical stability of **^{F}CMP-10**, approximately 50% of the immobilized peptides never discharged and were apparently bound permanently to the collagen films. This might represent the most stable fraction of the **^{F}CMP-10**–collagen complexes, comprising either a thermally stable collagen domain or a complex composed of 2 CMPs and 1 collagen strand. Similar sustained release profiles for **^{F}CMP-6**, **-7**, and **-10** were observed when CMPs were loaded onto a preformed 3D collagen gel (Figure S2), suggesting that this approach has potential applications in collagen gel-based drug delivery. Because the 5-carboxyfluorescein (5CF) conjugated onto our CMPs for release experiments is small (Mw: 376.33 Da) and hydrophobic, much like small drug compounds, we predict that CMP conjugates can be used as general vehicles for drug delivery in both 2-D and 3-D collagen matrices, with CMP-7 being used for sustained drug release and CMP-10 for permanent attachment of bioactive molecules.

To evaluate the potential of our new modification technique in modulating cellular behavior on collagen scaffolds, we prepared a series of poly(ethyleneglycol)-CMP conjugates (PEG-CMPXs, Table 1) designed to reduce the cell adhesiveness when applied to prefabricated collagen film. Collagen is inherently a highly adhesive substrate for cell attachment and many pathological conditions are caused by uncontrolled migration and proliferation of cells through collagen scaffolds. In addition, the cell repelling activity of this polymer could be used for spatio-temporal control of cell morphology and growth in 2D and 3D collagen matrices, with the long-term goal of complex tissue engineering.

PEG-CMP7, **PEG-CMP8**, **PEG-CMP10**, and their fluorescently labeled analogues, ^{F}PEG-CMPXs, were synthesized either by reaction of full length CMP-7 with $^{(F)}$PEG$_{2000}$-*N*-hydroxysuccinimide (NHS) or by solid supported peptide synthesis of $^{(F)}$CMP-8 and -10 from PEG$_{5000}$ preloaded resins. Conjugation of the large PEG unit interferes with the CMP's triple helix folding process resulting in suppression of the CD melting temperatures by 4~8 °C (Table 1). **^{F}PEG-CMP7** (T_m: 28 °C), **^{F}PEG-CMP8** (T_m: 38 °C) and **^{F}PEG-CMP10** (T_m: 65 °C) solutions were first heated to 45, 55, and 80 °C, respectively, and added separately to the collagen films in a culture plate sitting at room temperature as described before. Their IDIs are shown in Table 1. While the IDI of **^{F}PEG-CMP-7** and **-8** were an order of magnitude higher than those of **^{F}CMP-7** and **-8**, the IDI of **^{F}PEG-CMP10** and **^{F}CMP-10** were similar. It seems that large PEG unit interferes with the collagen binding process for short CMPs but for longer **^{F}PEG-CMP10**, the strong affinity between collagen and peptide seems to dominate the IDI value.

All three PEG-CMP conjugates tested exhibited sustained release behavior when incubated in 37 °C solution (Figure 5). The release was highest for **^{F}PEG-CMP7** (80% released within 3 days), followed by **^{F}PEG-CMP8** (65% released within 3 days) and **^{F}PEG-CMP10** (15% released in 3 days). The results are similar to those of **CMP-X**s where the order of sustainability correlated with the stability of CMPs. However, unlike **^{F}CMP-10**, the release profile of **^{F}PEG-CMP10** was continual and showed no clear indication to suggest the presence of peptides permanently bound to the collagen films. Although conjugation of PEG to the **^{F}CMP-10** did not affect the IDI value determined at room temperature, the release profile conducted at 37 °C strongly suggests that PEG conjugation destabilizes the peptide–collagen complex. Nevertheless, the results show that, for the most part, the CMP binding affinity and sustained release characteristics remain active even after conjugation of large hydrophilic polymers to the peptide end.

To test the ability of PEG-CMP to repel live cells on a collagen matrix, type I collagen films treated with the PEG-CMPs in the same manner as described above were seeded with human breast epithelial cells (MCF-7) at a cell density of 5.6×10^5 cells/mL. Initially, cells only attached to the unmodified areas of collagen films, and even after a three-day incubation at 37 °C, the areas treated with PEG-CMPXs were devoid of cells even though the cells formed a confluent layer on nontreated areas (Figure 5, inset). Continued incubation at 37 °C eventually led to invasion of cells into PEG-CMP modified areas within 5, 7, and 9 days for **PEG-CMP7** (Figure S3), **-8** (Figure S3) and **-10** (Figure 5, inset), respectively. Comparing these results with the $^{(F)}$PEG-CMPX release profiles (Figure 5) indicates that cell invasion occurred when the PEG-CMP density was reduced to between 21 and 100 nmol/cm^2. These values are comparable to the 25 nmol/cm^2 density of chemically conjugated PEG on collagen film reported to be effective in repelling fibroblasts and bacterial cell attachment.[10]

In an attempt to augment the collagen binding affinity of a PEG-CMP without the use of longer CMPs that require hot application conditions damaging to collagens, we synthesized a four-arm PEG-CMP with each PEG arm (MW of each PEG arm: ~500 Da) terminating with **CMP-8**. We expected that this compound would exhibit relatively low T_m, similar to that of the unmodified **CMP-8** (T_m: 44 °C) but, when applied to collagen film, would show high binding affinity due to the multiligand effect. Multiarm PEG-CMP, designated as **MPEG-CMP8** (Figure S4), and its fluorescently labeled derivative **^{F}MPEG-CMP8** were prepared by reaction of full length **CMP-8** or carboxyfluorescein conjugated **CMP-8** (see Experimental Section) with commercially available four-arm, NHS-activated PEG. The yield of the four-arm PEG and **CMP-8** coupling reaction, determined by ^{1}H NMR, was approximately 83%, which corresponded to an average of 3.3 **CMP-8**s per four-arm core PEG molecule. This was in agreement with the gel permeation chromatography trace

(Figure S5) that showed multiple distinct peaks with the highest peak and the second highest peak corresponding to the product comprising 3 and 4 **CMP-8**s, respectively. We were surprised to discover that the T_m of these compounds, 58 °C for **MPEG-CMP8** and 62 °C for **^{F}MPEG-CMP8**, were about 14~18 °C higher than that of the **CMP-8** (Table 1). Despite its large size, the core PEG unit served as a template that stabilized the intramolecular CMP triple helix. Such a template-induced stabilization effect has been observed only for small templates such as Kemp's triacid and tris(2-aminoethyl) amine (TREN) that can put the three CMP strands in close proximity.[47] Our results show for the first time that even a branched polymer with long flexible arms can be an effective template for the triple helix. When concentrated **^{F}MPEG-CMP8** solution (100 mg/mL) in deionized water was first heated above its T_m and then cooled to room temperature, a self-supporting hydrogel was formed (Figure S6). This hydrogel is a result of intermolecular CMP triplexes that physically polymerize the **^{F}MPEG-CMP8** into a massive network structure. The hydrogel slowly disintegrated when heated to 37 °C suggesting that the nontemplated, intermolecular CMP trimers are not as thermally stable as the templated intramolecular CMP trimers that melt at 62 °C.

Type I collagen film was treated with 80 °C **^{F}MPEG-CMP8** solution followed by excess washing with 4 °C buffer solution as described before. Almost 100% of the applied **^{F}MPEG-CMP8** was immobilized onto the collagen film in a form of a thick and clear hydrogel. Incubation at 37 °C led to disintegration of this hydrogel between day 3 and day 5, evidenced by visual disappearance of the hydrogel and the quick drop (60% loss) in the **^{F}MPEG-CMP8** content of the collagen film (Figure 5). Even after complete dissolution of the gel, approximately 40% of the **^{F}MPEG-CMP8** remained on the collagen film and was subsequently released in a sustained manner similar to the other ^{F}PEG-CMPXs. Cell binding results (Figure S3) were also consistent with the release profile as cell invasion took place between 5 (**MPEG-CMP8** density: 115 nmol/cm^2) and 7 (95 nmol/cm^2) days following cell seeding and incubation. The slower release profile of **$^{(F)}$MPEG-CMP8** compared to **$^{(F)}$PEG-CMP8** may be an indication of a mutiligand effect; however, the small difference makes us believe that collagen binding/release behavior is complicated by its tendency to form intramolecular complexes and self-supporting hydrogel that excludes collagen–CMP interactions.

Although the multiarmed PEG raised the thermal stability of the CMP trimer by acting as a template, it reduced the triple helix folding rate which provided an opportunistic time window for applying quenched, singled-stranded **MPEG-CMP8** solution to the collagen film. The triple helix refolding yield, determined by CD,[11,48–52] for a 57.5 μM solution cooled within 4 min from 80 °C to room temperature was approximately 71% for **CMP-8** but only 46% for **MPEG-CMP8**. The refolding yields remained the same even when the concentration of the solutions was raised up to 500 μM, the upper concentration limit for CD measurements. This suggested that, for the quenched room temperature **MPEG-CMP8** solution, about 54% of the CMPs were in single-stranded state available to interact with collagen. Indeed, when quenched **MPEG-CMP8** solution was applied to the collagen film, the initial binding after cold buffer wash was 48.4%. The modified collagen film also exhibited sustained release behavior (Figure 5) and cell repelling activity up to 3 days (Figure S3) demonstrating the effectiveness of collagen modification without concerns for collagen denaturation.

Conclusion

In tissue engineering and regenerative medicine, collagens are often used as a temporary ECM to support the formation and integration of functional tissue.[52] The latest developments in cell biology indicate that during tissue repair, many growth factors and

signaling molecules work as spatial and temporal regulators of cell behavior in tight association with the ECM.[53,54] We believe that our CMP-mediated collagen modification represents a novel effort to encode such spatio-temporal cues onto biologically derived scaffolds without compromising their biochemical features. We demonstrated that our CMPs have a specific binding affinity for collagen that is driven by triple helical propensity as indicated by nanoparticle localization and human liver tissue staining experiments. Such binding mechanism is unprecedented and may uncover the behaviors of a number of natural proteins that incorporate collagen-like sequences. We also discovered that the CMPs discharge from the collagen molecule in a sustained fashion that directly correlates with their helical propensity, and that they can easily form conjugates with bioactive molecules without adversely affecting their binding affinity. This modification technique can therefore potentially be used to attach multiple bioactive components such as adhesion molecules, growth factors, and antimicrobial agents to collagen scaffolds while allowing some degree of control over the relative concentration, spatial location, and release rate of the attached compounds. This technique could be readily applied to fabricate collagen-based implants that can be imaged or tracked for diagnostic purposes, can attract specific cells to defined locations within the implant, and release bioactive drugs to treat disease or minimize complications after surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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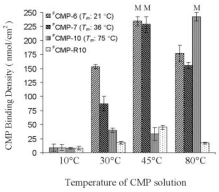

Figure 1.

Density of CMPs bound to collagen films (type I, bovine) after treatment with CMP solutions followed by extensive washing. CMP solutions preincubated at four different temperatures were applied to cell culture plates at room temperature coated with stable type I collagen film. The amount of CMP remaining on the collagen film was determined by fluorescence intensity of wash solutions. M marks the maximum binding condition for each CMP-X, which was used to determine initial dissociation index (IDI). Error bars represent ± SD.

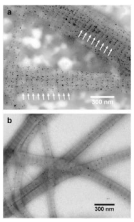

Figure 2.
Transmission electron micrographs of reconstituted type I collagen fiber from mouse tail tendon after incubation with gold nanoparticles displaying CysCMP-7 (a) or CysCMP-R7 (b). The white arrows indicate periodic positions of nanoparticles on collagen fibers located precisely at one interface between the dark and the white bands facing the collagen molecule's C-terminal end.

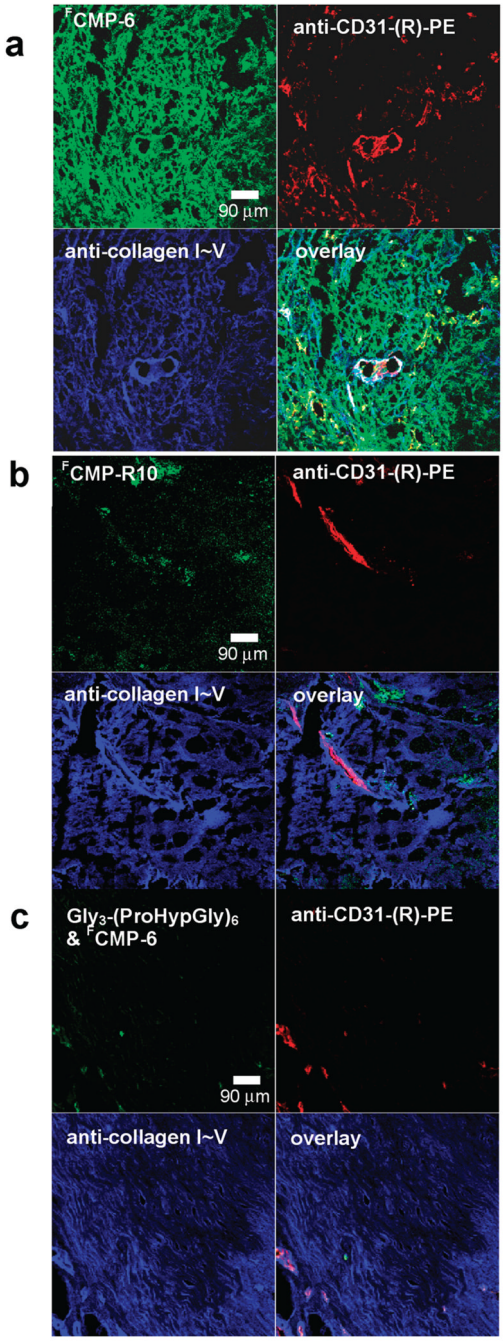

Figure 3.

^{F}CMP-6 binds specifically to collagens of human liver tissue. (a, b) Fluorescence confocal microscopy of human liver carcinoma stained with either ^{F}CMP-6 (green) (a) or ^{F}CMP-R10 (green) (b), and anti-CD31 mAb (red) and anticollagen I-V pAb (blue). Frozen, unfixed liver sections were thawed, washed with acetone and PBS, pH 7.4, and blocked with fetal bovine serum. A 50 μM solution of ^{F}CMP-6 or ^{F}CMP-R10 was then applied along with anti-CD31-(*R*)-PE and anticollagen I~V. The anticollagen antibody was visualized using antirabbit secondary antibodies conjugated to Alexa Fluor 647. As the overlay image indicates, the basement membrane and endothelial staining observed in ^{F}CMP-6 staining matches the pattern observed in anticollagen I~V staining (a); however, no discernible staining is

observed for ^{F}CMP-R10, whereas the pattern observed in the anticollagen I~V antibody is very clear (b). For the blocking experiment (c), liver sections were pretreated with 100 μM solution of GlyGlyGly-(ProHypGly)$_6$ before staining with ^{F}CMP-6 and antibodies as above. ^{F}CMP-6's affinity to collagens in the liver tissue is negated by the pretreatment with GlyGlyGly-(ProHypGly)$_6$.

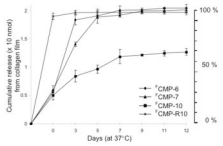

Figure 4.

Cumulative release profiles of ^{F}CMP-X from collagen films in 37 °C, PBS buffer solution (pH 7.4). Initially, 20 nmol of ^{F}CMP-X in PBS solution at predetermined temperature was applied to collagen film (type I bovine skin; area: 0.0616 cm^2). Day 0 represents ^{F}CMP-X released after extensive washing with 4 °C PBS buffer. Collagen films were incubated in 37 °C buffer solution and the concentrations of released ^{F}CMP-X were determined by measuring the UV–vis absorbance of buffer solutions at 493 nm. Data is reported as the mean ± SD of the four samples.

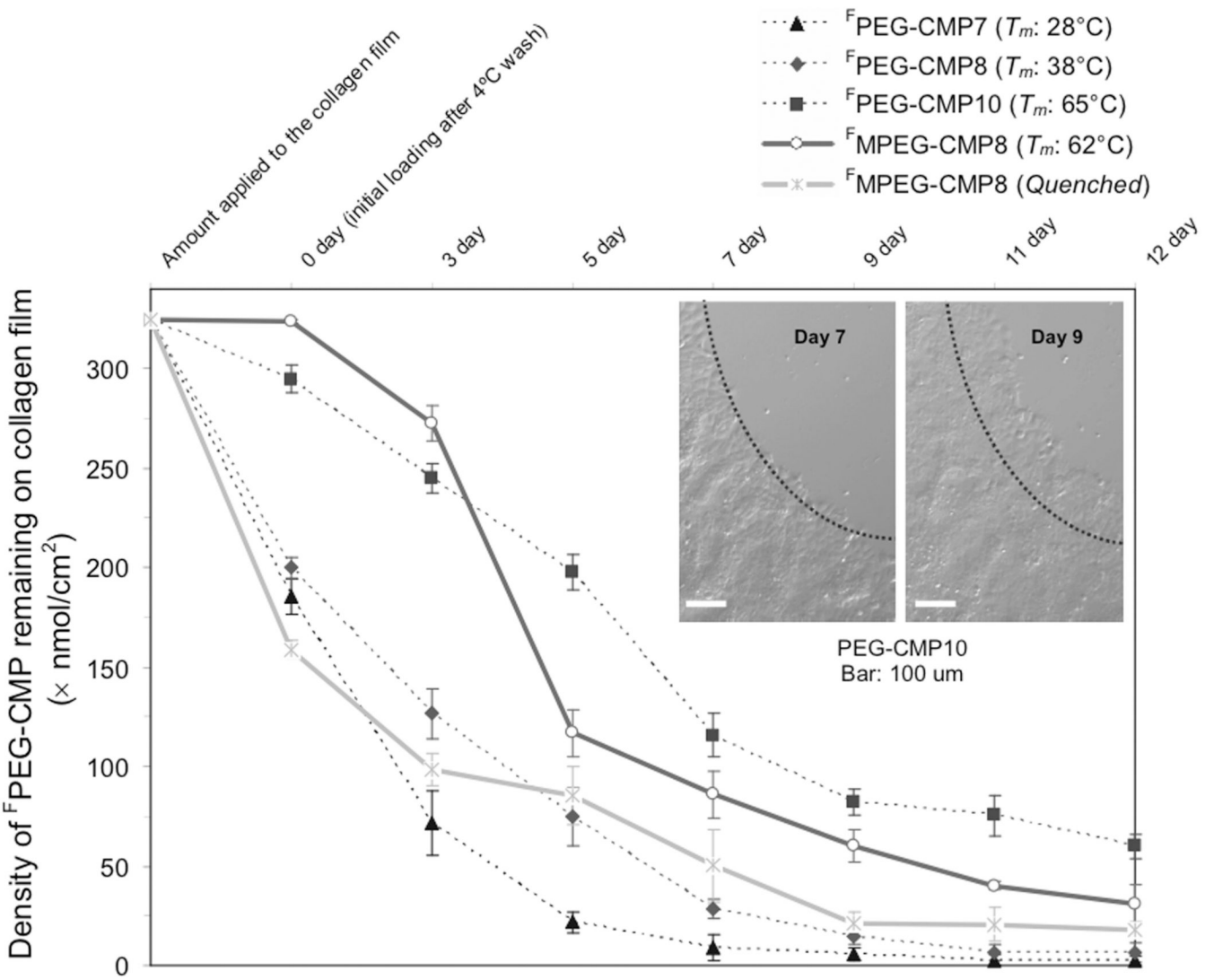

Figure 5.

Density profiles of ^{F}PEG-CMPXs and ^{F}MPEG-CMP8 remaining on collagen films during incubation at 37 °C, PBS buffer solution (pH 7.4). The charging and release conditions are the same as Figure 4. The quenched sample was first heated to 80 °C, cooled to 25 °C within 4 min, and immediately applied to the collagen film. Inset: optical micrographs of PEG-CMP10 treated collagen films seeded on day 0 with human breast epithelial cells (MCF-7) and incubated at 37 °C. The inset pictures correspond directly to the density profiles. Only the areas to the right side of the dotted line were treated and the whole collagen film was seeded with the cell suspension (5.6×10^5 cells/mL).

Table 1

Melting Transition Temperatures and Initial Dissociation Indices of Collagen Mimetic Peptide (CMP) Derivatives

CMP	sequence	T_m[a] (°C)	IDI[b] (M)
^{F}CMP-6	5CF-Gly_3-$(ProHypGly)_6$	21 ± 1	3.3×10^{-8}
^{F}CMP-7	5CF-Gly_3-$(ProHypGly)_7$	36 ± 1	1.7×10^{-8}
^{F}CMP-10	5CF-Gly_3-$(ProHypGly)_{10}$	75 ± 1	1.4×10^{-8}
^{F}CMP-R10[c]	5CF-Gly_3-random$Pro_{10}Hyp_{10}Gly_{10}$		1.2×10^{-6}
^{F}PEG-CMP7[d]	FL-PEG_{2000}-Gly_3-$(ProHypGly)_7$	28 ± 1	1.3×10^{-7}
^{F}PEG-CMP8[e]	5CF-Gly_3-$(ProHypGly)_8$-Gly_3-PEG_{5000}-OH	38 ± 1	1.0×10^{-7}
^{F}PEG-CMP10[e]	5CF-Gly_3-$(ProHypGly)_{10}$-Gly_3-PEG_{5000}-OH	65 ± 1	1.3×10^{-8}
PEG-CMP7[f]	mPEG_{2000}-Gly_3-$(ProHypGly)_7$	27 ± 1	
PEG-CMP8[e]	$(ProHypGly)_8$-Gly_3-PEG_{5000}-OH	38 ± 1	
PEG-CMP10[e]	$(ProHypGly)_{10}$-Gly_3-PEG_{5000}-OH	60 ± 1	
^{F}MPEG-CMP8[g]	multiarm PEG-Gly_3-$(ProHypGly)_8$-Lys(5CF)-$CONH_2$	62 ± 1	
MPEG-CMP8[g]	multiarm PEG-Gly_3-$(ProHypGly)_8$- $CONH_2$	58 ± 1	
CMP-8	Gly_3-$(ProHypGly)_8$-$CONH_2$	44 ± 1	
CysCMP-7	Cys-$(ProHypGly)_7$	41 ± 1	
CysCMP-R7[h]	Cys-random$Pro_7Hyp_7Gly_7$		

[a] Measured by circular dichroism spectroscopy in 57.5 μM, pH 7.4, PBS solution (Figure S7).

[b] IDI: initial dissociation index.

[c] 5CF-GGGGPPP^{H}P^{H}GPGGGPP^{H}PP^{H}GP^{H}GPP^{H}PGP^{H}P^{H}PGGP^{H}P^{H}PP, (P^{H}Hyp).

[d] FL-PEG_{2000}: fluorescein-PEG (average molecular weight: 2250 Da).

[e] PEG5000: average molecular weight, 5300 Da.

[f] mPEG2000: CH_3O-PEG-OH (average molecular weight: 2200 Da).

[g] Structure shown in Figure S4 (average molecular weight: 11000 Da).

[h] CGPGP^{H}PP^{H}PPGPPP^{H}GP^{H}P^{H}PP^{H}GP^{H}GG.