Supporting Information for

Direct detection of collagenous proteins by fluorescently labeled collagen mimetic peptides

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Materials and Methods

Materials

For peptide synthesis and labeling, Fmoc-Gly-OH, Fmoc-Pro-OH, H-Gly-OH, fluorenylmethyloxy chloroformate (Fmoc-Cl) and synthesis reagents including HBTU, DIPEA, NMP and trifluoroacetic acid (TFA) were purchased from Advanced ChemTech (Louisville, KY) and used without further purification. Fmoc-Hyp(tBu)-OH and PyBroP were purchased from EMD millipore (San Diego, CA). TentaGel R RAM resin was purchased from Peptides International (Louisville, KY). 5(6)-carboxytetramethylrhodamine (TAMRA) was purchased from Life Technologies (Carlsbad, CA). Piperidine, PyAOP, nitrobenzaldehyde, 5(6)-carboxyfluorescein (CF), triisopropylsilane (TIS) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

Acid soluble rat-tail type I collagen, bovine type II collagen, and mouse type IV collagen were purchased from BD Bioscience (San Jose, CA). Complement component C1q from human serum, fibronectin from bovine plasma, and laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane were purchased from Sigma-Aldrich. ProMMP-1, and p-aminophenylmercuric acetate were acquired from EMD millipore. Human umbilical vein endothelial cells (HUVECs) and EGM-2 cell growth media were obtained from Lonza (Walkersville, MD). Precast NuPAGE® Novex 4-12% Bis-Tris Gel (1.0 mm, 12 well), Novex® Sharp unstained protein standard, and SDS-PAGE running buffer, sample buffer, and other SDS-PAGE reagents were purchased from Life Technologies.

To prepare MMP-1 cleaved collagen chain fragments, proMMP-1 (1 μg) was activated by p-aminophenylmercuric acetate (1.1 mM) in 132.5 μL of TNC buffer (50 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 0.02% NaN_3, pH 7.5) at 37 °C for 3 hr, after which type I collagen (62.6 μg) was added to the activated MMP-1 solution and incubated at room temperature for at least 3 days.[1,2] To obtain the cell lysate, confluent HUVECs cultured in EGM-2 media at 37 °C were lysed with lysis buffer (25 mM Tris-HCl, 0.5% Triton X-100, 200 mM NaCl, 2 mM EDTA, phosphatase and protease inhibitors; Roche Applied Science). The lysate solution was spun at 15,000 g for 15 min and the supernatant was collected. The recombinant streptococcal collagen-like protein Scl2.28CL was expressed and characterized following the methods introduced by Mohs et al.[3]

For immunohistochemical staining, anti-collagen I antibody was purchased from Abcam (ab292, Cambridge, MA); bovine serum albumin (BSA), goat serum, and Triton X-100 were purchased from Sigma-Aldrich; DAPI was obtained from Roche Applied Science (Indianapolis, IN); Alexa fluor® 594 $F(ab')_2$ fragment of goat anti-rabbit IgG and Prolong® Gold antifade reagent were purchased from Life Technologies. The skin and cornea tissues were harvested from a 23 month-old wild-type C57BL/6 mouse. The tissues were fixed with 4% paraformaldehyde in PBS solution (pH 7.4) for 1 hr and cryopreserved in Tissue-Tek O.C.T. medium (Sakura Finetek, Torrance, CA). Cryosections of 8 μm thickness were obtained and mounted onto charged glass slides. Paraffin embedded mouse bone sections were a kind gift from Dr. Catherine Foss and Dr. Collin Torok at Johns Hopkins University School of Medicine. The leg containing the tibia bone was harvested from a 12-week-old female athymic nu/nu mouse. The muscle tissues were manually trimmed, and the bone was fixed in 10% neutral-buffered formalin at room temperature overnight. The bone was demineralized in Decal Stat solution (Decal Chemical, Suffern, NY) for 3 hr at room temperature, embedded in paraffin and sectioned to 4 μm at Tissue Microarray Facility of Johns Hopkins University. The formalin-fixed normal and fibrotic rat livers were a kind gift from Jie Yan and Yuzhan Kang at National University of Singapore. The tissues were sectioned to 10 μm thickness on glass slides and stained by Masson trichrome by the Reference Histology Laboratory, Johns Hopkins Medical Institutions.

Synthesis of fluorescently labeled collagen mimetic peptides

Non-caged CMPs were synthesized using Fmoc-mediated solid-phase chemistry by manual or automated synthesis as described previously.[2,4] The caged $^{NB}(GPO)_9$ was prepared by introducing

Fmoc(N-*o*-nitrobenzyl)Gly-OH (synthesized according to Tatsu et al.[2,5]) in the middle of the standard solid-phase peptide synthesis. The Hyp residue following the ^{NB}Gly was conjugated using 9 molar equiv of Fmoc-Hyp(tBu)-OH, 8.8 molar equiv of PyBroP, and 20 molar equiv of DIPEA for over 24 hr to overcome the low reactivity caused by the steric hindrance of the NB cage group.[2] The remaining sequence including the GGG spacer was completed by HBTU chemistry, followed by on-resin labeling with 6 molar equivalent of CF or TAMRA activated by 6 molar equivalent of PyAOP for over 24 hr.[6-8] The full length fluorescent CMPs were cleaved from resin by treating the resin with TFA/TIS/H_2O (95:2.5:2.5) for 3 hr and the cleaved peptide was purified by reverse phase HPLC on a Vydac C18 column using a linear gradient mixture of water (0.1% TFA) and acetonitrile (0.1% TFA) (5-45% acetonitrile gradient in 40 min). The purified peptides were analyzed by MALDI-TOF MS (Fig. S1): m/z calculated 2975.1 [M + Na^+] for $CF(GPO)_9$, found 2975.0 [M + Na^+]; m/z calculated 2975.1 [M + Na^+] for $CF^SG_9P_9O_9$, found 2973.5 [M + Na^+]; m/z calculated 3110.2 [M + Na^+] for $CF^{NB}(GPO)_9$, found 3109.4 [M + Na^+]; m/z calculated 2975.1 [M + Na^+] for $CF(GPO)_9$ [$CF^{NB}(GPO)_9$ after UV decaging], found 2974.1 [M + Na^+]; m/z calculated 3142.3 [M + H^+] for TAMRA-$^{NB}(GPO)_9$, found 3141.4 [M + H^+]; m/z calculated 3007.2 [M + H^+] for TAMRA-$(GPO)_9$, [TAMRA-$^{NB}(GPO)_9$ after decaging], found 3006.3 [M + H^+]. CD spectra and CD melting curves of the labeled CMPs were acquired in PBS solutions using a JASCO 715 CD spectrophotometer. The solutions were incubated for at least 24 hr at 4 °C prior to CD experiment to ensure folding (Fig. S2).

SDS-PAGE staining and imaging

Proteins were resolved on NuPAGE® Novex 4-12% bis-tris gels under denaturating conditions using an XCell SureLock™ Mini-Cell electrophoresis system (Life Technologies). NuPAGE® MOPS SDS running buffer containing 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, and 1 mM EDTA (pH 7.7) was used. Protein samples were heated to 85 °C in NuPAGE® LDS sample denaturing buffer for 10 min before the gel loading. Unless specified otherwise, 2 μg of protein was used for each lane. The gels were run at 200 V for 50-60 min. Reducing conditions were used only for gels carrying type IV collagen, HUVECs lysate, and C1q, for which NuPAGE® reducing agent was mixed with the protein samples, and 500 μL of NuPAGE® antioxidant was added to the cathode buffer before loading. After electrophoresis, the gels were washed by deionized water briefly three times to remove the remaining SDS. No fixation of the protein bands was performed, except for Scl2.28CL which was found to diffuse out of the gel easily without fixation; the gel carrying Scl2.28CL (shown in Fig. 3B) was fixed using 5% (wt) glutaraldehyde for 30 min and washed with deionized water before staining. PBS solutions (3-6 mL) containing 6 μM of $CF(GPO)_9$ (or $CF^SG_9P_9O_9$) were heated to 85 °C for 10 min and immediately pipetted onto the gels. The gels were soaked in this CMP solutions in dark under gentle shaking for 3 hr at room temperature, followed by washing with deionized water three times (1 hr each time) or overnight. The gels were then scanned using a Typhoon™ 9410 variable mode imager (Amersham Biosciences) under the settings of 488 nm blue laser excitation, 520 nm band-pass filter, 450 V of PMT, and 50 μm resolution. The dilution series of collagen I (Fig. 2A) was imaged using 600 V of PMT to examine the sensitivity limit of $CF(GPO)_9$. The fluorescence intensities of the protein bands were quantified using GE ImageQuant™ TL software. After fluorescence imaging, all gels were further stained with coomassie brilliant blue (CB) G-250 (Bio-Rad) for 2-3 hr followed by washing, and imaging under white light illumination using a Gel Doc EQ system (BioRad).

Immnunohistochemistry

Frozen fixed tissue slides were allowed to equilibrate to room temperature and dried under air flow. The tissue sections were permeabilized by cold methanol at -20 °C for 10 min, and incubated in 1×PBS solution. The paraffin embedded bone sections were de-paraffinized by two cycles of 5 min xylene wash, two cycles of 5 min 100% ethanol wash, and two cycles of 5 min 95% ethanol wash followed by soaking in 1×PBS solutions. Subsequently, to each slide, 0.5 mL of blocking solution (10% v/v goat serum and 0.3% Triton X-100 in 1×PBS) was added, and allowed to react for 30 min at room temperature.

$CF^{NB}(GPO)_9$, $CF^{S}G_9P_9O_9$ or TAMRA-$^{NB}(GPO)_9$ were dissolved in 1×PBS solution containing anti-collagen I antibody (1:100 dilution), 0.1% BSA and 0.3% Triton X-100. After blocking, CMP solutions of designated concentrations were applied to the tissue sections (100 μL for each slide) and allowed to incubate for 10 min. The CMP folding and collagen binding were triggered by exposing the samples to UV light (~15 mW/cm^2) for 5-12 min (incubation time depends on CMP concentration). After irradiation, the tissue sections were gently covered by parafilms to prevent drying and incubated in a humidity chamber at 4 °C for over 1.5 hr. The tissue slides were then washed by soaking in 1×PBS solutions for 5 min three times and probed by Alexa fluor® 594 F(ab')$_2$ fragment of goat anti-rabbit IgG (1:300) for 1 hr at room temperature to visualize the anti-collagen I antibody. When necessary, DAPI solutions (1 μg/mL) were applied to the slides for 60 s before the slides were washed by PBS again. The tissue slides were dried, mounted by Prolong® Gold antifade reagent and covered with glass cover slips. The stained sections were imaged by a Nikon Eclipse TE2000-E microscope (Nikon Instruments, Melville, NY). Ammonium acetate solutions (50 mM) containing 1 mM of $CuSO_4$ were applied directly to the tissue sections to reduce the background autofluorescence during imaging liver tissues.[9]

SI Figures

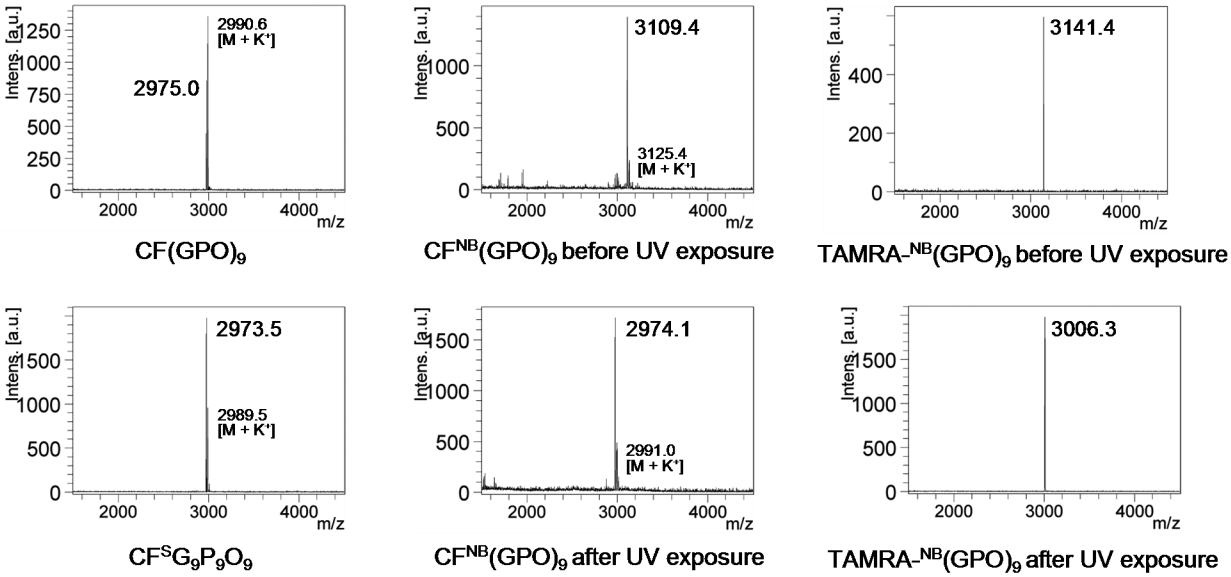

Figure. S1 MALDI-TOF Mass spectra of fluorescently labeled CMPs.

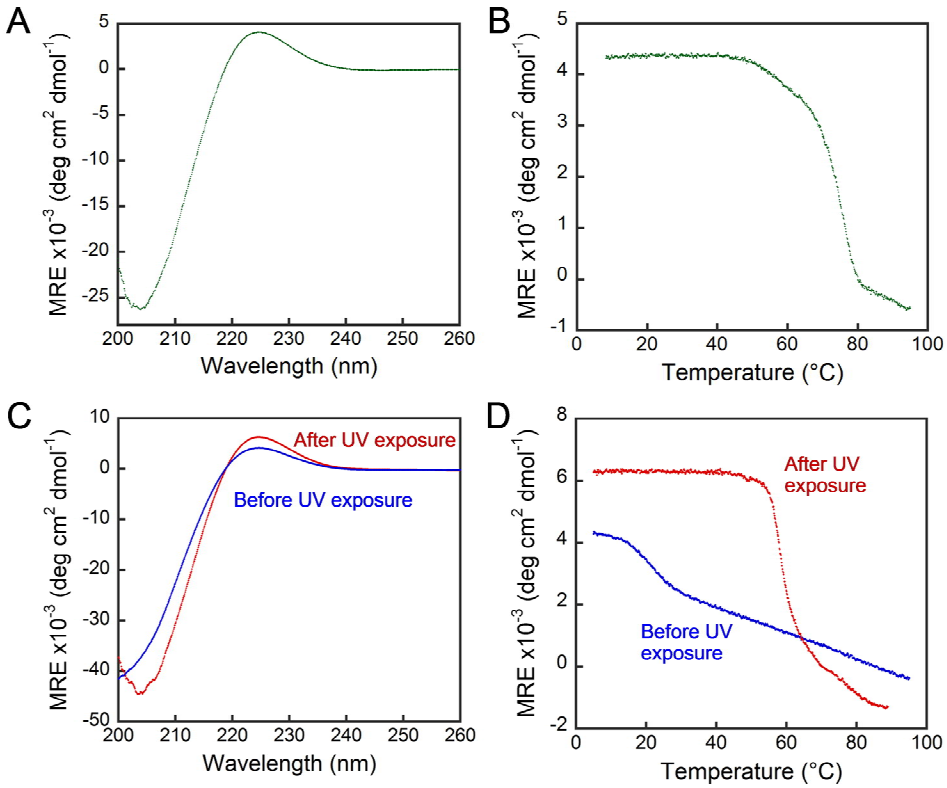

Figure. S2 CD studies of fluorescently labeled CMPs. (A) CD spectrum of CF(GPO)$_9$ measured at 4 °C. (B) CD melting curve of CF(GPO)$_9$ with a melting temperature (T_m) of 75 °C. The CD spectra (C) and melting curves (D) of TAMRA-^{NB}(GPO)$_9$ before and after UV exposure. The CD study demonstrates that the CMP regains its triple helical folding capacity after photo-cleavage of the NB cage group, similar to the photo-cleavage of NB of the CF^{NB}(GPO)$_9$.[2] All samples were dissolved in PBS solutions and incubated at 4 °C for at least 24 hr before CD measurement to ensure folding. All melting curves (in B, D) were generated by monitoring CD signals at 225 nm with a 1 °C/min heating rate.

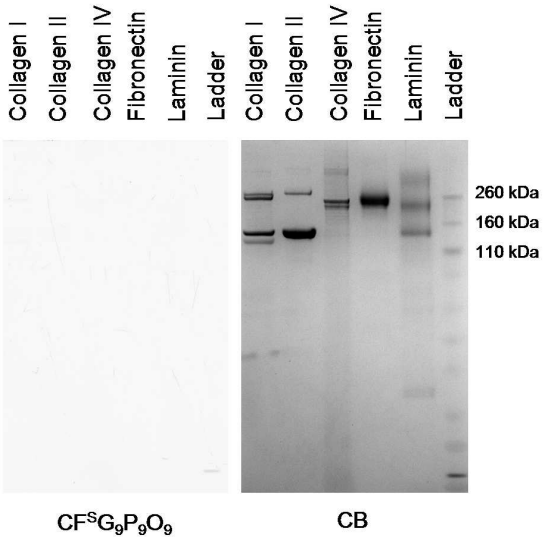

Figure. S3 SDS-PAGE of various types of collagens and ECM proteins (each lane is loaded with 2 μg of protein) stained by $CF^{S}G_9P_9O_9$ (left) and subsequently by commassie blue (right). The sequence-scrambled $CF^{S}G_9P_9O_9$ which lacks triple helical folding capacity showed virtually no binding affinity to the collagen bands.

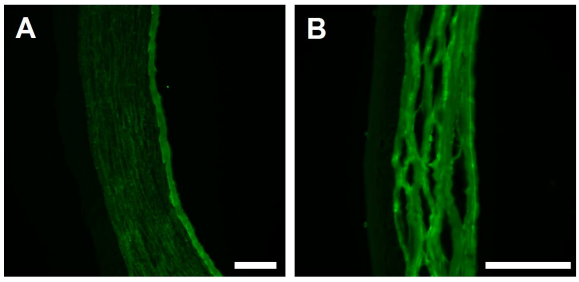

Figure. S4 Fluorescence micrographs of unfixed (A) and paraformaldehyde fixed (B) mouse cornea sections from the same animal, stained by 30 μM of photo-activated $CF^{NB}(GPO)_9$ (in green). Both sections were stained by the same procedure and imaged under similar conditions (A, exposure time: 1204 ms, gain: 1.3; B, exposure time: 878 ms, gain: 1.3). Significantly brighter intensity of the corneal stroma of the fixed sample (B) compared to the unfixed sample (A) suggests that chemical fixation causes collagen denaturation and enhances CMP binding (scale bars: 75 μm).

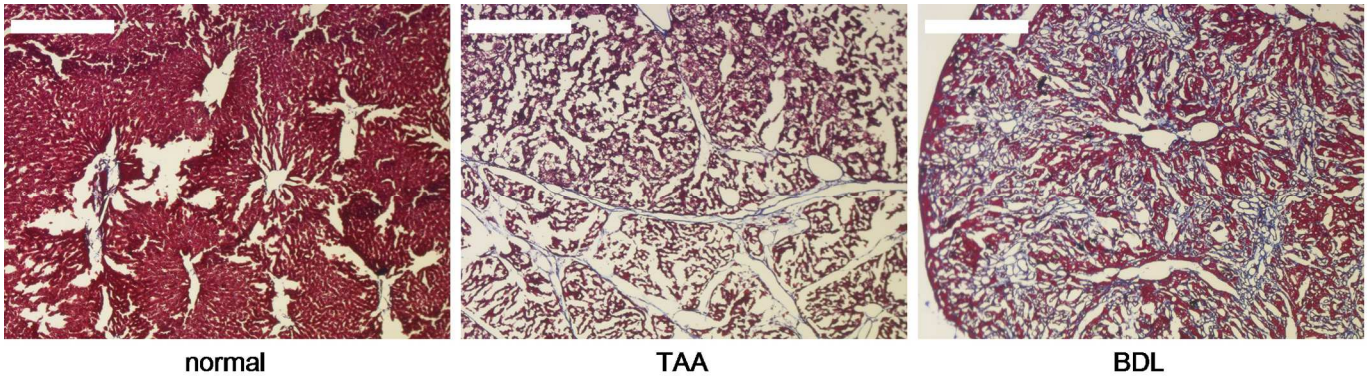

Figure. S5 Normal and fibrotic (TAA and BDL) liver tissue sections stained with conventional Masson Trichrome reagents. Collagen is stained in faint blue, and cells are stained in red. Collagen distributions in all three images are similar to their respective CMP stained images in Fig. 5 (scale bars: 0.5 mm).

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