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Supporting information

A single stranded fluorescent peptide probe for targeting collagen in connective tissues

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Materials and Methods

Materials

(2S,4S)-Fmoc-Amp(Boc)-OH was obtained from Top peptide Biotechnology Co. Ltd (Shanghai, China). Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Hyp(tBu)-OH, O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), 2-Chlorotrityl chloride resin (200~400 mesh, loading = 0.8 mmol/g) and Rink amide resin (200~400 mesh, loading = 0.8 mmol/g) were purchased from GL Biochemical Company (Shanghai, China). Diisopropylethylamine (DIEA) was obtained from Hanhong Chemical Technology Co. Ltd (Shanghai, China). (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) and 5(6)-Carboxyl fluorescein (FAM) were provided by Aladdin Industrial Corporation (Shanghai, China). Trifluoroacetic acid (TFA) and Triisopropylsilane (Tis) were purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). Modified Masson's Trichrome Stain Kit, bovine serum albumin (BSA) and goat serum were obtained from Solarbio Science & Technology Co. Ltd (Beijing, China). DAPI was purchased from Beyotime (Shanghai, China). All the commercial reagents were of analytical grade and were used without further purification.

Synthesis of peptide FCMP

Peptide FCMP with sequence FAM-(Gly-Pro-Hyp)$_7$-Gly was synthesized in-house by standard Fmoc solid phase peptide synthesis (SPPS) method using 2-Chlorotrityl chloride resin (0.8 mmol loading). Stepwise couplings of amino acids were performed using Fmoc-amino acids (4 eq.), HOBt (4 eq.), HBTU (4 eq.) and DIEA (6 eq.). Resin was washed by DMF (3×10 mL) and DCM (2×10 mL) after each step of coupling, and the Fmoc protecting group was removed with 20% (v/v) piperidine in DMF. Chloranil test

was used to check the status of coupling reaction and Fmoc deprotetction. After the completion of the synthesis of peptide sequence (Gly-Pro-Hyp)$_7$-Gly, the mixture of FAM (4 eq.), HOBt (4 eq.), HBTU (4 eq.) and DIEA (6 eq.) in DMF was added to the resin, and incubated for 16 hrs to conjugate FAM to the N-terminal of the peptide. The resin was treated with TFA/DCM/TIS (90:5:5) for 2.5 hrs to deprotect the side-chain protecting group and cleave the peptide from the resin. The peptide was harvested by precipitation with cold Et_2O. Crude products were collected by re-suspension in cold Et_2O, sonication and centrifugation, and were then purified using reverse phase HPLC on a C18 column. The peptide was lyophilized and stored at -20 °C for future use. The purity of the peptide was confirmed by mass spectrometry.

Synthesis of peptide ssFCMP

Peptide with sequence Ac-(Gly-Pro-Hyp)$_3$-Gly-Pro-Amp-(Gly-Pro-Hyp)$_3$-Gly-NH_2 (denoted as CMP-Amp) was synthesized in-house by similar Fmoc SPPS protocols using Rink amide resin (0.8 mmol loading). (2S,4S)-Fmoc-Amp(Boc)-OH was used in the middle of the synthesis for the conjugation of Amp. At the end of peptide synthesis, 25% Ac_2O in DMF was added to the resin, and incubated for 15 min in order to acetylate the N-terminal of the peptide. Peptide was cleaved from the resin using TFA/H_2O/TIS (95:2.5:2.5), and collected by precipitation with cold Et_2O. Crude products were obtained by re-suspension in cold Et_2O, sonication and centrifugation, and were then purified using reverse phase HPLC on a C18 column. The peptide was lyophilized and stored at -20 °C.

After FAM (4 eq.) was activated by PyAOP(4 eq.) in DMF, DIEA (6 eq.) was added, and they were mixed well by stirring in nitrogen atmosphere. The as-prepared peptide CMP-Amp (25 mg) was dissolved in 3 mL DMF. The peptide and FAM solutions were mixed and foil-wrapped to avoid the loss of fluorescence. The mixture was stirred constantly in an ice bath for 48 hrs to complete the reaction. Finally, the product was repeatedly dialyzed against ultrapure water to remove excessive FAM and other remaining reagents. The fluorescence intensity of FAM in the dialysis buffer after exchange was monitored to assure the complete removal of FAM in the solution. The

peptide ssFCMP was lyophilized and stored at -20 °C for future use. The purity of the peptide was confirmed by mass spectrometry.

Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectra were recorded on an Aviv model 400 spectrophotometer (Applied Photophysics Ltd, England). Cells with a 2 mm-path length were used, and a Peltier temperature controller was applied to regulate the temperature. Peptide Solutions of 50 μM ssFCMP and FCMP were prepared in 10 mM phosphate buffer at pH 7.4, respectively. Wavelength scans were performed from 190 to 280 nm with a 0.5 nm increment per step and a 0.5 s averaging time. Each measurement was repeated three times.

Thermal stability was determined by monitoring the amplitude of the characteristic CD peak at 225 nm as a function of increasing temperature from 4 °C to 75 °C. The average heating rate was 0.5 °C/min and the equilibration time was 2 min. The peptides were equilibrated for at least 24 hrs at 4 °C prior to the melting experiments. The melting temperature (Tm) was defined as the temperature at which the fraction folded is equal to 0.5 in the trimer to monomer transition.

UV-Vis and Fluorescence Assays

UV-Vis spectra were recorded on a UV-1750 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Peptide Solutions of 15 μM ssFCMP and FCMP were prepared in 10 mM phosphate buffer at pH 7.4 for UV measurements, respectively. Fluorescence spectra were measured on a RF-5301PC fluorescence spectrometer equipped with a Xenon lamp as an excitation source (Shimadzu Corporation, Kyoto, Japan). Peptide Solutions of 350 μM ssFCMP and FCMP were prepared in 10 mM phosphate buffer at pH 7.4 for fluorescence characterization, respectively. Fluorescence measurements were conducted from 505 to 650 nm with a 1 nm increment per step at an excitation wavelength of 497 nm. Both peptides were heated at 85 °C for 30 min to ensure complete unfolding. Then the refolding process of the peptides was immediately monitored by real-time measuring the fluorescence intensity at 525 nm for 75 min at 15 °C.

Colorimetric measurements

Photographs were obtained with a digital camera (Canon SX220 HS) for peptides ssFCMP and FCMP with a concentration of 350 μM under three different conditions: incubation at 4 °C, heating at 85 °C for 30 min, and re-equilibration at 4 °C for > 24 hrs after the heating at 85 °C.

Fluorescence imaging of collagen fibers using ssFCMP

Type I collagen solution with a concentration of 1 mg/mL was prepared in 0.5 M acetic acid, and was dialyzed against 20 mM NaCl solution to generate collagen fibers. 40 μL peptide solution of 50 μM ssCMP in phosphate buffer (10 mM, pH 7.4) was added to the collagen fibers in phosphate buffer (80 μL, pH 7.4). The mixture was incubated for 3 hrs and excessive peptide was eliminated by washing the fiber with water three times. 5 μL of the peptide/fiber mixture was dropped onto a glass slide and pressed with cover glass. Bright-field and fluorescence microscopy images of ssFCMP-stained collagen fibers were recorded on a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany). The exposure time for bright-field and fluorescence microscopy measurements was 600 ms and 495 ms, respectively. Fluorescence micrographs were obtained using ultraviolet and blue light as excitation source. As a control, FAM was used to stain collagen fibers similarly, and microscopy images were recorded accordingly.

Histological tissue staining using ssFCMP

The tendon, tail, eye and ear tissues were obtained from 9-week old rats. 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. The tissues were sectioned to 4 μm thickness on glass slides. Frozen fixed tissue slides were air-dried at room temperature. The tissue sections were permeabilized by cold methanol at -20 °C for 10 min, and incubated in 20 mM PBS. Each slide was then treated with 0.5 mL blocking solution (5% v/v goat serum in PBS), and incubated at room temperature. Bibulous paper was used to remove the blocking solution from the slides.

Solutions of fluorescent probe peptides ssFCMP and FCMP with a concentration of 15 μM were prepared in 10 mM phosphate buffer (pH 7.4), respectively. 100 μL of the peptide solution was applied to the tissue sections, covered with parafilm, and incubated at 4 °C for 2 hrs. The parafilm was then removed and the solution on the slides was absorbed by bibulous paper. Subsequently, 100 μL DAPI solution (10 μg/mL) was applied to the tissue section for 1 min at room temperature. The slides were washed by 10 mM phosphate buffer for 5 min three times. A drop of mounting medium (resinene) was added on the tissue slide, and a glass cover slip was used to cover the slide. The stained tissue sections were imaged on a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany). The rat ear tissue sections were examined using both ssFCMP and Masson's Trichrome Staining.

Inhibition experiments

Solution of a peptide inhibitor $G(POG)_{10}$ with a concentration of 15 μM was prepared in 10 mM phosphate buffer (pH 7.4), and was heated at 85 °C for 30 min to achieve the unfolded state. 100 μL of the peptide solution was applied to the rat tendon tissue section, covered with parafilm, and incubated at 4 °C for 2 hrs. The parafilm was then removed and the solution on the slide was absorbed by bibulous paper. The slides were washed by 10 mM phosphate buffer for 5 min three times. The tissue section was then stained with 100 μL ssFCMP (15 μM), covered with parafilm, and incubated at 4 °C for 2 hrs. Subsequently, 100 μL DAPI solution (10 μg/mL) was applied to the tissue section for 1 min at room temperature. The slides were washed by 10 mM phosphate buffer for 5 min three times. A drop of mounting medium (resinene) was added on the tissue slide, and a glass cover slip was used to cover the slide. The stained tissue sections were imaged on a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany).

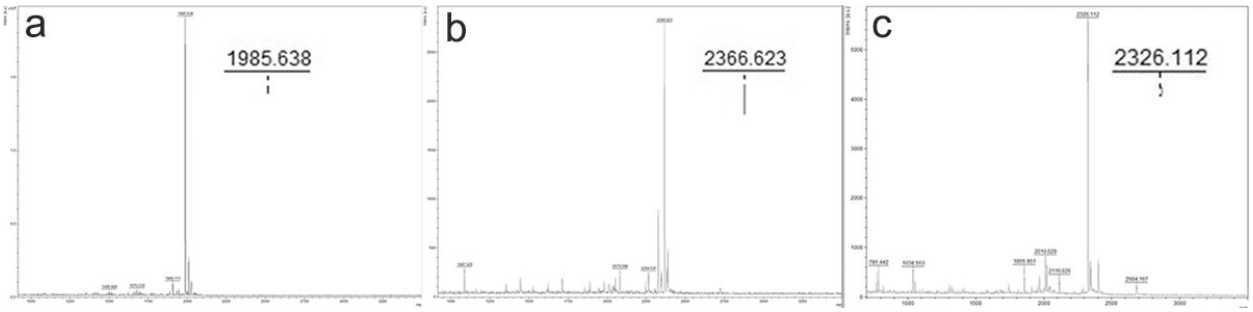

Figure S1. MALDI-TOF Mass spectra of peptides CMP-Amp (a), ssFCMP (b) and FCMP (c). m/z calculated 1985.927 $[M+H]^+$ for CMP-Amp, found 1985.638 $[M+H]^+$; m/z calculated 2366.958 $[M+Na]^+$ for ssFCMP, found 2366.623 $[M+Na]^+$; m/z calculated 2325.922 $[M+Na]^+$ for FCMP, found 2326.112 $[M+Na]^+$.

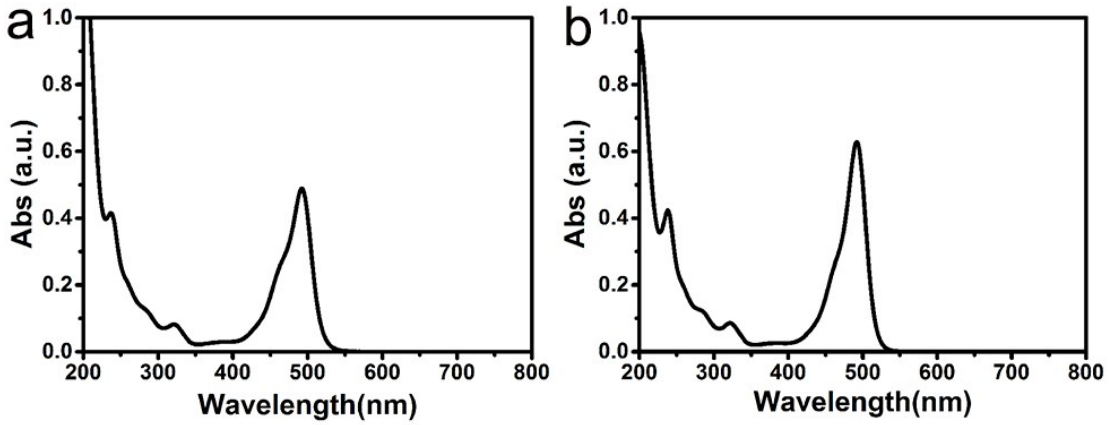

Figure S2. UV-Vis spectra of peptides FCMP (a) and ssFCMP (b). Both peptides were prepared at the same concentration of 15 μM in 10 mM phosphate buffer (pH 7.4).

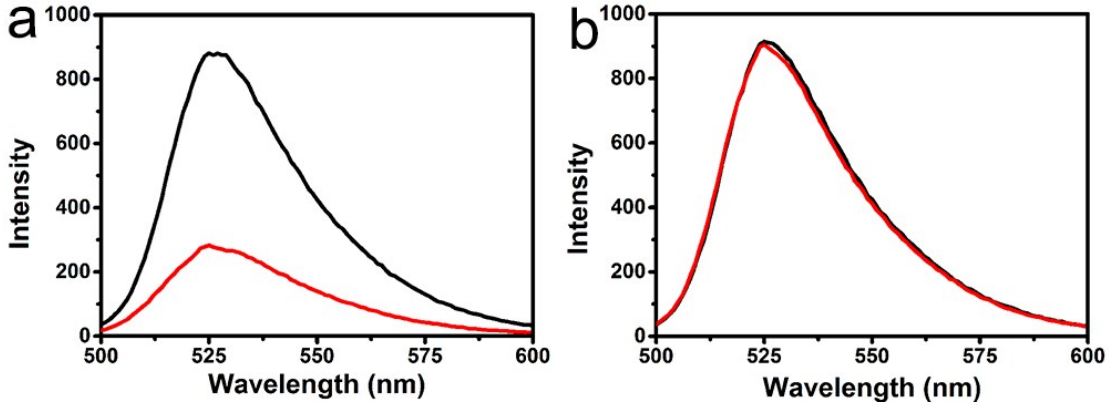

Figure S3. Fluorescence emission spectra of peptides FCMP (a) and ssFCMP (b) at an excitation wavelength of 497 nm. Fluorescence spectra were measured for both peptides immediately after heating at 85 °C for 30 min (black) and after 75 min of refolding at 15 °C (in red). Both peptides were prepared at the same concentration in 10 mM phosphate buffer (pH 7.4) for comparison.

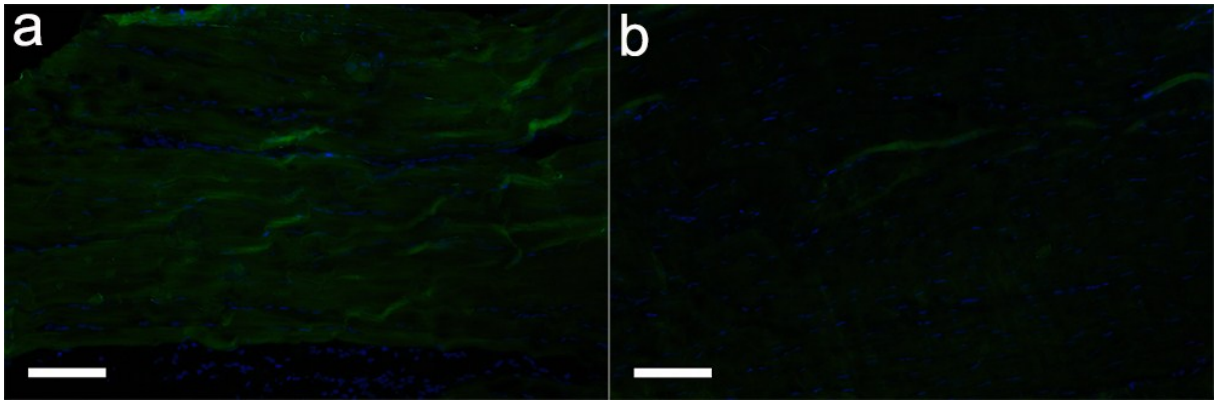

Figure S4. Fluorescence micrographs of rat tendon tissue sections stained with ssFCMP in the absence (a) and presence of a peptide inhibitor $G(POG)_{10}$ (b). The presence of $G(POG)_{10}$ blocks the ssFCMP staining of target collagen (in green). The tissue sections were co-stained with DAPI for cell nucleus (in blue). Scale bar = 100 μm.

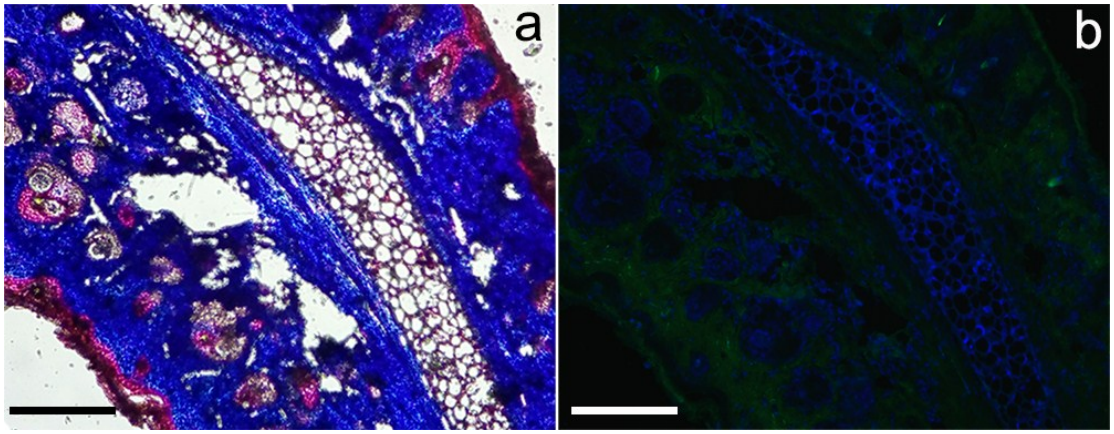

Figure S5. Histological tissue staining using Masson's Trichrome (a) and probe peptide ssFCMP (b). Bright field micrograph of rat ear tissue section using Masson's Trichrome stain (collagen in blue) (a); Fluorescent micrograph of the same rat ear tissue section

stained with ssFCMP for collagen (in green) and co-stained with DAPI for cell nucleus (in blue) (b). Scale bar=200μm.

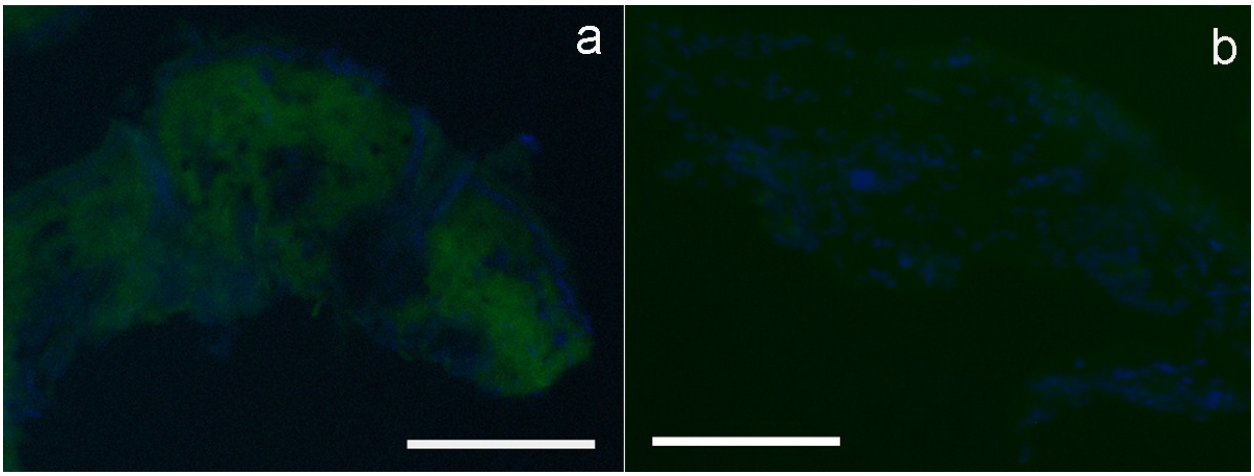

Figure S6. Histological tissue staining using probe peptide ssFCMP. Fluorescent micrographs of rat ear tissue sections stained with ssFCMP (a) and FCMP (b) (in green), and co-stained with DAPI (in blue). Peptides ssFCMP and FCMP with the same concentration of 350 μM were stored at 4 °C for two weeks after the heating at 85 °C for 30 min, and they were diluted to 15 μM to stain the tissue sections. Scale bar = 100 μm.