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ARTICLE

Highly specific recognition of denatured collagen by fluorescent peptide probes with the repetitive Gly-Pro-Pro and Gly-Hyp-Hyp sequences

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Denatured collagen is a key biomarker involved in various critical diseases such as cancer. Peptide probes with the repetitive $(Gly\text{-}Pro\text{-}Hyp)_n$ sequences have recently been found to selectively target denatured collagen; however, thermal or UV pretreatment is required to drive the peptides into the monomer conformation, which poses a substantial challenge for clinical applications. We herein have constructed two peptide probes FAM-GOO and FAM-GPP consisting of the repetitive $(Gly\text{-}Hyp\text{-}Hyp)_8$ and $(Gly\text{-}Pro\text{-}Pro)_8$ sequences, respectively. The CD, fluorescent and colorimetric studies have consistently revealed that FAM-GOO showed strong capability to form triple helical structure, while FAM-GPP pronouncedly displayed the single stranded conformation at temperatures as low as 4 ℃. The binding experiments have indicated that both peptide probes could recognize denatured collagen with high specificity, and FAM-GPP remarkably did not need the preheating treatment. The tissue staining results have shown that preheated FAM-GOO and unheated FAM-GPP could target denatured collagen in a broad variety of rat frozen and human FFPE tissue sections. Compared with antibodies specific for a certain type of collagen, both FAM-GOO and FAM-GPP are broad-spectrum probes for selectively detecting denatured collagen of different types and from different species. Importantly, FAM-GPP possessed the unique capability to maintain the monomer conformation by itself, thus avoiding the potential risks of the thermal or UV pretreatment. This novel peptide probe provides a handy and versatile biosensor for specifically targeting denatured collagen, which has attractive potential in the diagnosis and therapeutics of collagen-involved diseases.

Introduction

Collagen has been well known as a key biomarker involved in a plethora of critical diseases.[1-4] As the major component of the extracellular matrix, collagen plays a fundamental role in cell proliferation, differentiation and migration as well as other physiological processes such as tissue remodeling and organ morphogenesis.[5-10] The imbalance between the production and degradation of collagen has been widely reported to result in pathological conditions.[11-13] Over-expression of type I collagen has been observed in breast cancer and medulloblastoma.[14] The increasing degradation of type II collagen has been identified as a crucial pathogenic step in osteoarthritis.[15, 16] Furthermore, Abnormal remodeling of type IV collagen has been correlated with liver and lung fibrosis, cancer invasion and metastasis, and cardiovascular diseases.[17-20] The construction of robust collagen assays is therefore crucial for deciphering the molecular mechanism as well as developing new theranostics for these diseases.

A number of peptide probes have been discovered to target native collagen in recent decades. A peptide WREPSFCALS derived from the collagen binding domain of von Willebrand Factor (vWF) displayed binding affinity for collagen type I.[21] A few type I collagen-targeting peptides such as TKKTLRT and LRELHLNNN have been identified by exploring collagen-binding proteins.[22-24] Meanwhile, phage display libraries have been built to screen peptides targeting collagen. Peptide KLWVLPK was selected to specifically bind to collagen type IV in tumors, while peptide WYRGRL was found to bind type II collagen in articular cartilage.[25-30] Due to the tightly-bound, triple helical structure of collagen, these peptide probes unfortunately often suffer from severe drawbacks such as low affinity and nonspecific binding.[31]

Specific recognition of denatured or partially degraded collagen has obtained increasing interest since the discovery of collagen hybridizing peptides. Denatured collagen has been widely found in diseases such as cancer, arthritis and osteoporosis.[32-35] Recent studies have revealed that peptide probes with the repetitive amino acid sequences $(Gly\text{-}Pro\text{-}Hyp)_n$ (n=6-10) could bind to the partially unfolded segment of denatured collagen to reform the triple helical structure.[36] These collagen hybridizing peptides show highly selective recognition of denatured collagen only when they are in the monomer state. However, the $(Gly\text{-}Pro\text{-}Hyp)_n$ sequences possess a

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very strong tendency to form triple helical structure by themselves, and thermal pretreatment is required to drive the peptides into the monomer conformation.[36] This process makes it implausible to determine the exact concentration of the peptide probe in the monomer form, posing a substantial challenge for clinical applications.

Extensive efforts have been carried out to improve the design of collagen hybridizing peptides in order to curb their trimerizing capability. A photo-cleavable nitrobenzyl (NB) group was applied to modify the central glycine in peptide $(GPO)_9$, whose steric hindrance led to the prohibition of the homotrimer formation.[37] A fluorescent dye 5(6)-Carboxyfluorescein (FAM) was conjugated to a central unnatural amino acid (2S,4S)-aminoproline (Amp) in peptide $(GPO)_3$-GP-Amp-$(GPO)_3$, and the bulky FAM dye effectively hindered the homotrimer formation of the peptide.[38] Though the side-chain modifications of the $(GPO)_n$ sequences helped the peptides maintaining the monomer state, they also raised obstacles such as synthetic complexity, potential toxicity and difficulties in precise quantitation of pure monomers.

In order to discover more potent collagen-targeting peptide sequences, we have herein constructed two peptide probes FAM-GOO and FAM-GPP composed of repetitive $(Gly\text{-}Hyp\text{-}Hyp)_8$ and $(Gly\text{-}Pro\text{-}Pro)_8$ sequences, respectively. We have revealed that both constructed peptide probes could selectively recognize denatured collagen in a wide variety of rat and human connective tissues. Remarkably, FAM-GPP maintained the monomer conformation by itself, avoiding the potential damage due to the thermal pretreatment. These two novel peptide probes provide robust broad-spectrum biosensors for denatured collagen, with promising applications in various collagen-involved diseases.

Experimental Section

Materials and methods

Rink amide resin (200~400 mesh, loading = 0.8 mmol/g), Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Hyp(tBu)-OH, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and 1-Hydroxybenzotriazole (HOBt) were purchased from GL Biochemical Company (Shanghai, China). N,N-Diisopropylethylamine (DIEA) was obtained from Hanhong Chemical Technology Co. Ltd (Shanghai, China). 5(6)-Carboxylfluorescein (FAM), piperidine, N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 2-(N-Morpholino)ethanesulfonic acid (MES) were provided by Aladdin Industrial Corporation (Shanghai, China). Trifluoroacetic acid (TFA) and Triisopropylsilane (Tis) were purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). Bovine serum albumin (BSA) and goat serum were obtained from Solarbio Science &Technology Co. Ltd (Beijing, China). DAPI was purchased from Beyotime (Shanghai, China). Hemoglobin, trypsin, and pepsin were obtained from Yuanye Biological Technology Co.Ltd (Shanghai, China). Albumin from Human Serum (HSA) was provided by BoMei Biotechnology Co.Ltd (Hefei, China). Gelatin was obtained from Biotopped Science & Technology Co.Ltd (Beijing, China). All the commercial reagents were of analytical grade and were used without further purification.

Peptide synthesis

Peptides $(GPO)_8G$, $(GPP)_8G$, $(GOO)_8G$, FAM-$(GPO)_8G$ (FAM-GPO), FAM-$(GPP)_8G$ (FAM-GPP), and FAM-$(GOO)_8G$ (FAM-GOO) were synthesized in-house by standard Fmoc solid phase synthesis (SPPS) method. Fmoc-amino acids (4 eq.), DIEA (6 eq.), HBTU (4 eq.) and HOBt (4 eq.) were applied during each step of amino acid coupling on rink amide resin. Fmoc protection groups were eliminated by 20% piperidine in DMF after washing the reaction mixture with DMF and DCM. The status of coupling and deprotection reactions was monitored by the proguanil test. For FAM-labeled peptides, FAM was conjugated to the N-terminal of the peptide by using FAM (4 eq.), HOBt (4 eq.), HBTU (4 eq.) and DIEA (6 eq.). After the completion of peptide synthesis, the resins were treated with TFA/TIS/H_2O (95:2.5:2.5) for 3 hrs to remove the tBu groups and release the peptide. The peptides were harvested by precipitation with cold Et_2O. Crude products were collected after re-suspension in cold Et_2O, sonication and centrifugation. The peptides were purified using reverse phase HPLC on a C18 column, and the purity of the peptides were confirmed by mass spectrometry. m/z calculated 2235.4 $[M+Na]^+$ for $(GPO)_8G$, found 2236.2 $[M+Na]^+$; m/z calculated 2340.4 $[M]^+$ for $(GOO)_8G$, found 2341.2 $[M]^+$; m/z calculated 2107.4 $[M+Na]^+$ for $(GPP)_8G$, found 2108.4 $[M+Na]^+$; m/z calculated 2570.7 $[M]^+$ for FAM-$(GPO)_8G$, found 2571.1 $[M]^+$; m/z calculated 2698.7 $[M]^+$ for FAM-$(GOO)_8G$, found 2699.0 $[M]^+$; m/z calculated 2442.7 $[M]^+$ for FAM-$(GPP)_8G$, found 2443.0 $[M]^+$.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were collected by a Chirascan CD spectrophotometer (Applied Photophysics, UK) equipped with a Peltier temperature controller. CD samples were prepared at a concentration of 300 μM in 10 mM PBS buffer at pH 7.4. Wavelength scans were performed from 190 nm to 260 nm with a 0.5 nm increment per step at 4 °C. Peptides were heated at 90 °C for 20 min and then re-equilibrated at 4 °C for at least 24 hrs prior to the measurement. The thermal stability of peptides was measured by monitoring the amplitude of the CD signal at 225 nm with an average heating rate of 0.2 °C min^{-1}. The melting temperature was defined as the extrema of the first derivative of the thermal transition curves. Each scan was repeated three times.

UV-Vis and fluorescence spectroscopy

The UV-Vis spectra were measured on a UV-1750 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Solutions of peptides FAM-GPO, FAM-GOO, and FAM-GPP were prepared in 10 mM PBS (pH 7.4) at a concentration of 300 μM. After heating at 80 °C for 20 minutes, the peptide solutions were diluted to 15 μM for UV measurement. Fluorescence spectra were recorded on an RF-5301PC fluorescence spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a xenon lamp as the excitation source. Solutions of peptides FAM-GPO, FAM-GOO, and FAM-GPP were prepared in 10 mM PBS (pH 7.4), heated at 80 °C for 20 min, and incubated at 4 °C for 2 days prior to the fluorescence experiments. Fluorescent spectra were recorded for each peptide under three conditions: equilibrated at 4 °C, heated to 80 °C for 20 minutes, and re-equilibrated at 4 °C for 24 hrs after heating at 80 °C. The excitation wavelength was 493 nm. The refolding process of FAM-

GPO, FAM-GOO and FAM-GPP were monitored by measuring the fluorescence intensity at 4 °C at 533 nm for 60 minutes immediately after heating at 80 °C for 20 min.

Colorimetric measurements

Photographs of solutions of peptides FAM-GPO, FAM-GOO, and FAM-GPP with a concentration of 300 μM were obtained under three conditions: equilibrated at 4 °C, heated to 80 °C for 20 minutes, and re-equilibrated at 4 °C for 24 hrs after heating at 80 °C.

Protein binding assay

Gelatin (5 mg/mL) was prepared in 10 mM PBS (pH 7.4) at 70 °C. 6 uL of gelatin was added into each well of a 96-well plate, and incubated at 4 °C for 20 minutes to complete gelation. 100 uL solutions of 2 mM NHS and 10 mM EDC in 50 mM MES buffer (pH 4.7) were added and incubated overnight at 4 °C under gentle shanking to cross-link the gelatin hydrogel films. The cross-linked films were washed with 400 μL 10 mM PBS (pH 7.4) for 10 min three times. 50 μL solution of 20 μM fluorescent peptide probe (FAM-GPO, FAM-GOO or FAM-GPP) in 10 mM PBS (pH 7.4) was added to each well, and incubated at 4 °C for 5 hrs to allow its binding to the gelatin films. In the "Heating +" group, peptide solutions were heated at 80 °C for 15 min and quenched in ice water for 30 s immediately before the addition to the wells. In the "Heating -" group, peptide solutions were stored at 4 °C, and directly used without any heating pretreatment. The wells were then washed with 400 μL 10 mM PBS (pH 7.4) for 10 min three times. Fluorescence (ex: 493 nm, em: 533 nm) was recorded on an Infinite M200 (TECAN Corporation, Switzerland). Each measurement was repeated three times.

The binding assay of denatured collagen, HSA, BSA, hemoglobin, pepsin, and trypsin was carried out following similar protocols. Type I collagen (100 μg/mL) was dissolved in 0.5 M acetic acid solution, and heated at 70 °C for 15 minutes to allow denaturation. Solutions of HSA, BSA, hemoglobin, pepsin, and trypsin with a concentration of 100 μg/mL were prepared in 10 mM PBS (pH 7.4). The protein solutions were added into the wells of a 96-well plate, and air-dried. Solutions of peptide FAM-GPP were directly added without any preheating, while solutions of peptides FAM-GOO or FAM-GPO were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage. 50 μL solution of 20 μM fluorescent peptide probe (unheated FAM-GPP, heated FAM-GPO or heated FAM-GOO) in 10 mM PBS (pH 7.4) was added to each well, and incubated at 4 °C for 5 hrs to allow binding. The wells were washed with 400 μL 10 mM PBS (pH 7.4) for 10 min three times. Fluorescence (ex: 493 nm, em: 533 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each binding experiment was repeated three times.

Binding affinity of the peptide probes

Gelatin (10 μg/mL) was prepared in 10 mM PBS (pH 7.4) at 70 °C. 100 uL of gelatin was added into each well of a 96-well plate, and and air-dried. After coating, the gelatin film was washed with 400 μL 10 mM PBS (pH 7.4) for 3 min three times. 100 μL solution of BSA in 10 mM PBS (pH 7.4) (1% v/v) was added and incubated at room temperature for 1 hr to block non-specific binding. Solutions of peptide FAM-GPP were directly added without preheating, while solutions of peptides FAM-GOO and FAM-GPO were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage. 100 μL solution of 1 nM, 10 nM, 50 μM, 100 μM, 500 nM, 1 μM, 2 μM, 3 μM, 4 μM, 5 μM and 6 μM peptide probe (unheated FAM-GPP, heated FAM-GPO and heated FAM-GOO) in 10 mM PBS (pH 7.4) was added to each well, and incubated at 4 °C for 10 hrs to allow binding. The wells were washed with 400 μL 10 mM PBS (pH 7.4) for 10 min three times. Fluorescence (ex: 493 nm, em: 533 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each binding experiment was repeated three times. The Kd value was calculated by fitting the Kd equation using Origin software.

In vitro Cytotoxicity of the peptide probes

In vitro cytotoxicity of peptide probes FAM-GPO, FAM-GOO and FAM-GPP was evaluated by examining the viability of HeLa cells using Cell Counting Kit-8. Briefly, HeLa cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. 100 μL of HeLa cells was added to a 96-well cell-culture plate, and incubated at 37 °C for 4 hrs to allow attachment. 100 μL solution of peptide probes was added into the 96-well plate with four different final concentrations (0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL). The cell cultures were then kept incubated in the humidified atmosphere with 5% CO_2 at 37 °C for 48 hrs. 100 μL of 10% CCK8 was added to each well, and incubated for 1 hr. The OD value of each well was measured by a microplate reader. Cell viability was calculated as the mean absorption value of three measurements of each condition divided by the mean absorption value of the control group.

Rat tissue staining and imaging

All animal experiments were carried out with protocols approved by the ethics committee of Lanzhou University No.1 Hospital. The tendon, cornea, bladder, stomach, large intestine, ear, and kidney tissues were obtained from nine-week-old rats. These tissue slides were fixed with 4% paraformaldehyde in 1x PBS buffer for 1 hr and cryopreserved in Tissue-Tek O.C.T. medium. Half of the tissues were treated with 1% SDS (Sodium dodecyl sulfate) for 24 hrs under gentle shaking in order to denature collagen. The SDS-treated tissues were then rinsed with deionized water by a cycle of 30 min 3 times, 12 hrs and 30 min 3 times. Both normal and SDS-treated tissues were cryosectioned to 4 μm thickness, and mounted on charged glass slides. 0.5 mL solution of goat serum in PBS (5% v/v) was added onto each slide, and incubated at room temperature for 30 min to block non-specific binding. The blocking solution was removed with paper towel. A half of the solutions of peptide probes FAM-GPO, FAM-GOO and FAM-GPP were heated at 80 °C for 15 min and quenched in ice water for 30 s prior to the usage, while the half were stored at 4°C and used directly without any preheating. 100 μL solutions of heated and unheated peptide probes (15 μM) in 10 mM PBS (pH 7.4) were applied to cover the tissue sections of each slide. The slides were then covered with parafilm to prevent drying, and incubated at 4 °C for 4 hrs. After staining, the slides were immersed in 1x PBS buffer in a staining tank for 5 min, and repeated five times to wash off unbound staining agents. The stained rat tissue sections were imaged on a fluorescence microscope (phYFP channel, Zeiss, Germany).

For the inhibition experiments, the solution of inhibitor peptide $G(POG)_{10}$ was added onto the tendon tissues prior to staining the tissue slides with the fluorescent peptide probes. The solution was heated at 85 °C for 30 min to ensure the monomer state of the inhibitor peptide. The tissue slides were incubated at 4 °C for 4 hrs to allow complete binding of collagen with the inhibitor. The tissue slides were then washed with 1x PBS buffer five times to remove any unbound inhibitors.

Pathological human tissue staining and imaging

The following procedure was approved by the ethics committee of Lanzhou University No.1 Hospital. FFPE (formalin-fixed paraffin-embedded) tissues of human liver fibrosis, leiomyoma, liver cancer, stomach cancer, lung cancer, esophageal cancer, rectal cancer, and breast cancer were provided by the First Hospital of Lanzhou University. Paraffin was removed by rinsing with xylene (10 min x 3), 100% ethanol (5 min x 2), 95% ethanol (5 min x 2), 80% ethanol (5 min x 2), and deionized water (10 min x 2) in consecutive order. 0.5 mL solution of goat serum in PBS (5% v/v) was added onto each slide, and incubated at room temperature for 30 min to block non-specific binding. The blocking solution was removed with paper towel. 100 μL solutions of 15 μM peptide probe FAM-GOO (heated) and FAM-GPP (unheated) in 10 mM PBS (pH 7.4) were applied to cover the tissue sections of each slide. The slides were then covered with parafilm to prevent drying, and incubated at 4 °C for 4 hrs. After the staining of peptide probes, the parafilm was removed, and the excess solution was wiped away by paper towel. 200 μL of DAPI (5 μg/mL) solution in 10 mM PBS was applied to each tissue slide, and incubated at room temperature for 1 min. After DAPI staining, the slides were immersed in a staining tank in 10 mM PBS buffer for 5 minutes 5 times to wash off unbound DAPI. Pathological collagen (FAM channels) and nuclei (DAPI channels) in the tissue slides were imaged on a fluorescence microscope (Zeiss, Germany).

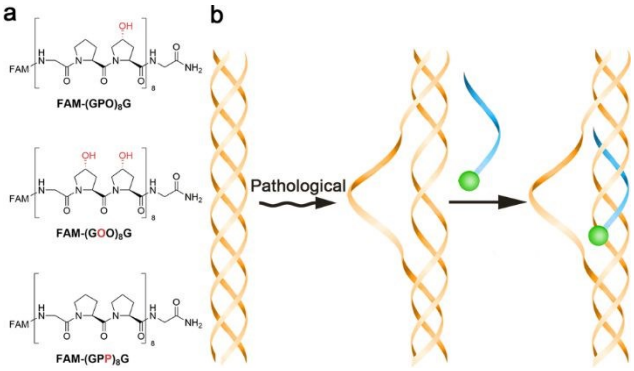
Results and discussion

Design of collagen-targeting fluorescent peptide probes

Peptide probes with the repetitive $(Gly\text{-}Pro\text{-}Hyp)_n$ sequences have recently been found to specifically target denatured collagen when they are in the monomer conformation.[37] Pro and Hyp are the most frequent residues in the X and Y positions of the triple helical domain of native collagen, respectively, while Gly-Pro-Hyp is considered as the most stabilizing triplet for triple helical structure. In order to discover novel collagen-targeting peptide probes, we have herein constructed two peptide probes FAM-GPP (with amino acid sequence $(Gly\text{-}Pro\text{-}Pro)_8$-Gly) and FAM-GOO (with sequence $(Gly\text{-}Hyp\text{-}Hyp)_8$-Gly), while FAM is a commonly used fluorescent dye 5(6)-Carboxyfluorescein. A peptide probe FAM-GPO (with sequence $(Gly\text{-}Pro\text{-}Hyp)_8$-Gly) consisting of the well-characterized $(Gly\text{-}Pro\text{-}Hyp)_n$ was synthesized as the control. Due to the similarity of Pro and Hyp, peptide probes FAM-GPP and FAM-GOO contain totally the same main chain as FAM-GPO, suggesting they may possess similar collagen-targeting capability (Scheme 1). In normal tissues, collagen is considered to maintain intact triple helical structure, while in pathological tissues, some unfolded sites are exposed in collagen.[37] Similarly as FAM-GPO, we propose that the novel peptide probes FAM-GPP and FAM-GOO could specifically hybridize with and recognize the unfolded chains in pathological collagen (Scheme 1).

Triple helix propensity of the fluorescent peptide probes

Triple helix propensity is a key index of collagen-targeting peptide probes, since they can only bind to collagen in the monomer state. CD spectra indicated that peptides GPO (with amino acid sequence $(Gly\text{-}Pro\text{-}Hyp)_8$-Gly) and GOO (with sequence $(Gly\text{-}Hyp\text{-}Hyp)_8$-Gly) displayed positive maximum absorption at 225 nm, which was characteristic of triple helical structure (Figure 1a). The CD melting experiments showed that peptides GPO and GOO formed triple helix with a melting temperature of 42 °C and 49 °C, respectively (Figure 1b-c). Notably, the absence of the characteristic peak at 225 nm and the linear thermal unfolding curve demonstrated that peptide GPP could not form a triple helical structure at temperatures as low as 4 °C (Figure 1a, S1). Peptide GOO possessed a slightly higher triple helical stability than GPO, suggesting that the Hyp in the X position may facilitate additional hydrogen bonding, thus enhancing the triple helix stability. In contrast, the replacement of Hyp in the Y position likely eliminated the critical water-bridged hydrogen bonds, thus totally disrupting the triple helix structure.[39]



Scheme 1. Schematic illustration of the constructed fluorescent peptide probes (a) targeting denatured collagen (b).

The triple helix propensity of the FAM-labeled peptide probes FAM-GPO, FAM-GPP and FAM-GOO were further examined by fluorescence spectroscopy (Figure 1d-f). UV-visible spectra indicated that all three peptide probes showed strong absorption at 493 nm (Figure S2). Fluorescence spectra of peptide probe FAM-GPO were recorded at an excitation wavelength of 493 nm under three conditions: equilibrated at 4 °C (black), heated to 80 °C for 20 minutes (red), and re-equilibrated at 4 °C for 24 hrs after heating at 80 °C (blue) (Figure 1d). FAM-GPO displayed weak fluorescence at 4 °C, since the peptide was in the triple helical conformation, and the close proximity of the three chains resulted in fluorescence self-quenching of the fluorophore FAM. It displayed much stronger fluorescence at 80 °C, since the transition to the monomer structure led to fluorescence recovery, which was consistent with previous reports.[40] The overlapped fluorescence spectra at 4 °C prior to and after heating indicated that FAM-GPO could completely reform the triple helix structure by heating and cooling steps.

Peptide probe FAM-GOO showed a similar fluorescence pattern as FAM-GPO (Figure 1e). FAM-GOO displayed weak fluorescence at 4 °C, strong fluorescence at 80 °C, and equally poor fluorescence after heating and cooling, indicating that FAM-GOO possessed

strong capability to fold into triple helix structure at low temperature. In contrast, peptide FAM-GPP displayed completely different fluorescence profiles from FAM-GPO and FAM-GOO (Figure 1f). The spectra of FAM-GPP were totally the same under all three conditions, indicating that FAM-GPP always maintained the monomer conformation (Figure 1f). The refolding capability of the three peptide probes were further examined by real-time monitoring of the fluorescent intensity at 533 nm at 4 °C immediately after heating at 80 °C for 20 min (Figure 1g). It indicated that peptides FAM-GPO and FAM-GOO could refold into triple helix within 60 min of cooling, while FAM-GPP did not possess any refolding ability.

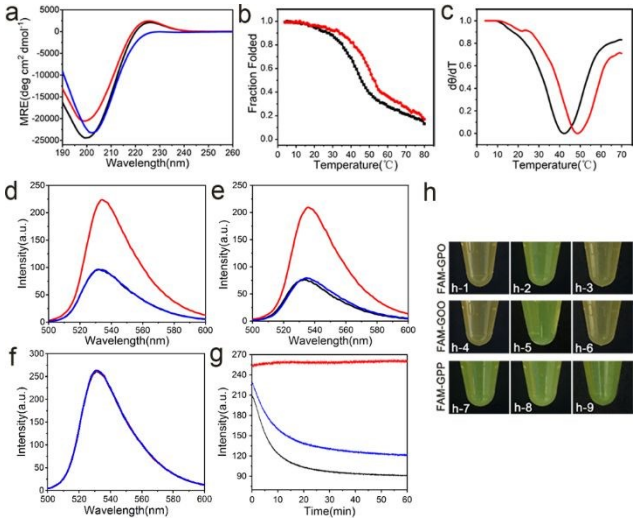


Figure 1. Triple helical propensity of the fluorescent peptide probes. (a) CD spectra of peptides GPO (black), GOO (red) and GPP (blue). (b) CD melting curves of peptides GPO (black) and GOO (red). (c) The first derivative of the melting curves of peptides GPO (black) and GOO (red). Fluorescence spectra of peptide probes FAM-GPO (d), FAM-GOO (e) and FAM-GPP (f) at an excitation wavelength of 493 nm under three conditions: equilibrated at 4 °C (black), heated to 80 °C for 20 minutes (red), and re-equilibrated at 4 °C for 24 hrs after heating at 80 °C (blue). (g) Real-time monitoring the fluorescence intensity of peptides GPO (black), GOO (red) and GPP (blue) at 533 nm at 4 °C immediately after heating at 80 °C for 20 min. (h) Visual changes of peptide solutions of FAM-GPO (h-1, 2, 3), FAM-GOO (h-4, 5, 6) and FAM-GPP (h-7, 8, 9) at 4 °C (h-1, 4, 7), heated to 80 °C for 20 min (h-2, 5, 8) and re-equilibrated at 4 °C (h-3, 6, 9).

The triple helix folding of the three peptide probes were also characterized by colorimetry (Figure 1h). The FAM dye has been observed to display different colors in the triple helical versus single stranded conformation.[40] Peptide FAM-GPO was in the triple helical structure when equilibrated or re-equilibrated at 4 °C, and it displayed the same peru color (Figure 1h-1, 3). When heated at 80 °C, FAM-GPO became unfolded into monomer, and appeared in green color (Figure 1h-2). Similarly as FAM-GPO, FAM-GOO showed peru color at 4 °C, which turned into green when heated to 80 °C (Figure 1h-4-6). In contrast, FAM-GPP always showed green color at 4 °C and 80 °C, confirming its single stranded conformation at both temperatures (Figure 1h-7-9). To conclude, all the CD, fluorescent and colorimetric results consistently demonstrated that peptide probes FAM-GPO and FAM-GOO possessed strong capability to form triple helix structure, while FAM-GPP always maintained the monomer state.

Collagen-targeting capability of the fluorescent peptide probes

The collagen-targeting capability of the three fluorescent peptide probes was evaluated using binding experiments (Figure 2). Preheated and unheated peptide probes were added onto gelatin films, and the unbound probes were removed. Preheated FAM-GPO and FAM-GOO showed strong binding affinity toward gelatin, while unheated FAM-GPO and FAM-GOO indicated little gelatin-binding ability (Figure 2a). It was consistent with previous reports that monomer conformation was a prerequisite for these peptide probes to target denatured collagen. Meanwhile, both preheated and unheated FAM-GPP displayed strong binding affinity toward gelatin, indicating that preheating was not necessary for FAM-GPP to bind with denatured collagen (Figure 2a). Furthermore, the equilibrium dissociation constant (Kd) of the peptide probes (preheated FAM-GPO, preheated FAM-GOO and unheated FAM-GPP) for gelatin was determined as 587.5 nM, 909.8 nM and 654.5 nM, respectively, indicating that peptide probes FAM-GOO and FAM-GPP displayed slightly less binding affinity toward denatured collagen than FAM-GPO (Figure S3).

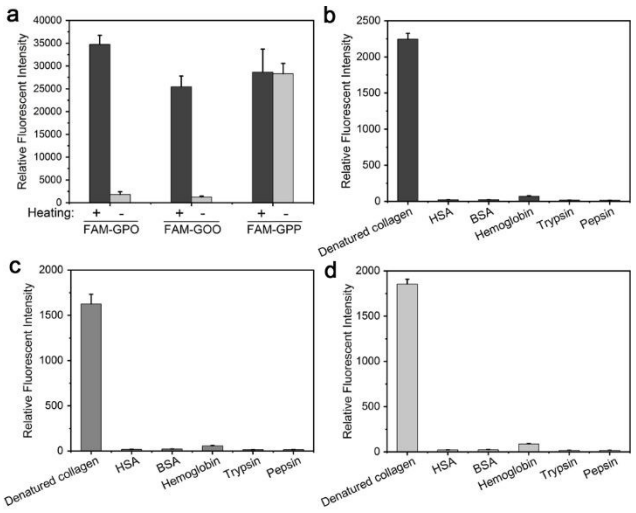


Figure 2. The binding specificity of the fluorescent peptide probes. (a) The binding affinity of peptide probes FAM-GPO, FAM-GOO and FAM-GPP toward gelatin films in a 96-well plate. Peptide solutions were preheated at 80 °C before their additions into the wells in the "Heating +" group, while they were stored at 4 °C and directly used in the "Heating -" group. The binding affinity of preheated FAM-GPO (b), preheated FAM-GOO (c) and unheated FAM-GPP (d) with denatured type I collagen and other proteins (HSA, BSA, hemoglobin, trypsin, and pepsin). The peptides probes were incubated in the wells coated with these proteins. Fluorescence intensity at an excitation wavelength of 493 nm and an emission wavelength of 533 nm was recorded.

The binding specificity of the fluorescent peptide probes (preheated FAM-GPO, preheated FAM-GOO and unheated FAM-GPP) was further examined (Figure 2b-d). Wells of a 96-well plate were coated with thermally denatured type I collagen, HSA, BSA, hemoglobin, trypsin and pepsin, respectively, and their binding affinity with the three peptide probes were measured. Preheated FAM-GPO, preheated FAM-GOO and unheated FAM-GPP all

showed strong binding with denatured collagen, and they displayed almost no binding with all other proteins (Figure 2b-d). These results demonstrated that both novel peptide probes FAM-GOO and FAM-GPP could recognize denatured collagen with high specificity, and FAM-GPP remarkably did not need any pretreatment prior to the binding assays. In vitro cytotoxicity of peptide probes FAM-GPO, FAM-GOO and FAM-GPP was evaluated by examining the viability of HeLa cells (Figure S4). All the peptde probes showed similarly high cell viability at various concentrations, demonstrating that they were highly biocompatible.

Tissue staining by the fluorescent peptide probes

The tissue staining capability of the three peptide probes were compared (Figure 3). Normal and SDS-treated rat tendon tissues were stained with preheated and unheated FAM-GPO, FAM-GOO and FAM-GPP, respectively (Figure 3). For normal tissues, the fluorescence micrographs indicated no staining by all the three peptide probes no matter preheated or not, suggesting that these peptide probes could not bind to intact collagen (Figure 3a-f). For SDS-treated tissues, the fluorescence micrographs clearly showed that FAM-GPO and FAM-GOO could recognize denatured collagen when they were preheated, but could not when unheated (Figure 3g-j). Notably, both preheated and unheated FAM-GPP could stain denatured collagen well (Figure 3k-l). These results demonstrated that both peptide probes FAM-GOO and FAM-GPP possessed similar capability as FAM-GPO to specifically stain denatured collagen in tissues, while FAM-GPP distinctly did not need any heating pretreatment.

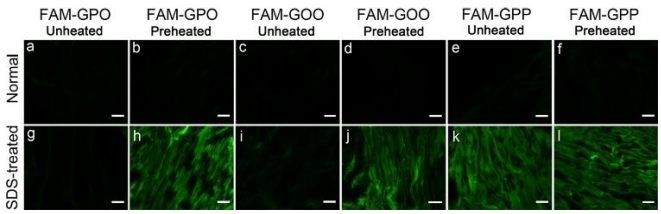


Figure 3. Fluorescence micrographs of normal (a-f) and SDS-treated (g-l) rat tendon tissues stained with unheated FAM-GPO (a, g), preheated FAM-GPO (b, h), unheated FAM-GOO (c, i), preheated FAM-GOO (d, j), unheated FAM-GPP (e, k), preheated FAM-GPP (f, l). Scale bar = 100 μm.

Inhibition experiments were performed using peptide G(POG)10 as an inhibitor to evaluate the binding pattern of the three peptide probes FAM-GPO, FAM-GOO and FAM-GPP (Figure S5). Peptide $G(POG)_{10}$ was applied onto the tendon tissues prior to the staining with the fluorescent peptide probes. In the presence of $G(POG)_{10}$, all the fluorescence micrographs of the stained tendon tissues showed very weak fluorescence, indicating that $G(POG)_{10}$ efficiently blocked the staining of denatured collagen by all three peptide probes (Figure S5). Probe peptides composed of $(GPO)_n$ sequences have been revealed to bind with the unfolded chains in denatured collagen.[41] Similarly as FAM-GPO, peptide probes FAM-GOO and FAM-GPP probably also hybridize with the unfolded sites and specifically target denatured collagen (Scheme 1).

Peptide probes FAM-GOO (preheated) and FAM-GPP (unheated) were further applied to stain SDS-treated rat cornea, bladder, stomach, intestine, ear, and kidney tissue sections (Figure S6-7). The fluorescence micrographs of all the stained tissues showed strong green fluorescence, demonstrating the robustness of both peptide probes to target denatured collagen in a broad variety of connective tissues (Figure S6-7). Compared with FAM-GPO and FAM-GOO, FAM-GPP possessed the unique capability to maintain the single stranded state, avoiding the preheating treatment.

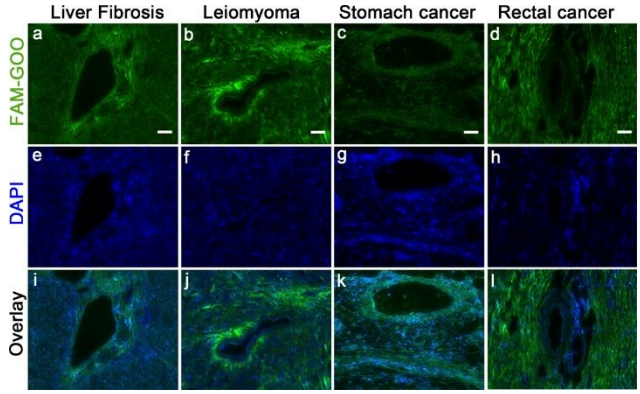


Figure 4. Pathological human tissue staining using preheated FAM-GOO. Fluorescence micrographs of liver fibrosis (a, e, i), leiomyoma (b, f, j), stomach cancer (c, g, k) and rectal cancer (d, h, l) tissue sections stained with preheated FAM-GOO (in green) and DAPI (in blue). Scale bar = 100 μm.

Human pathological tissue staining by the fluorescent peptide probes

Denatured collagen has been correlated with a wide range of pathological conditions such as fibrosis and tumors. The applicability of peptide probes FAM-GOO and FAM-GPP to specifically target denatured collagen in human pathological tissues were further explored (Figure 4-5, S8-9). Fluorescence micrographs of FFPE (formalin-fixed paraffin-embedded) tissues of human liver fibrosis, leiomyoma, stomach cancer, rectal cancer, esophageal cancer, breast cancer, liver cancer and lung cancer stained with preheated FAM-GOO all showed strong green fluorescence, indicating the versatility of FAM-GOO to detect denatured collagen in various pathological tissues (Figure 4, S8). The co-staining of DAPI for cell nucleus confirmed the distinctive collagen distribution in different types of diseased connective tissues (Figure 4, S8).

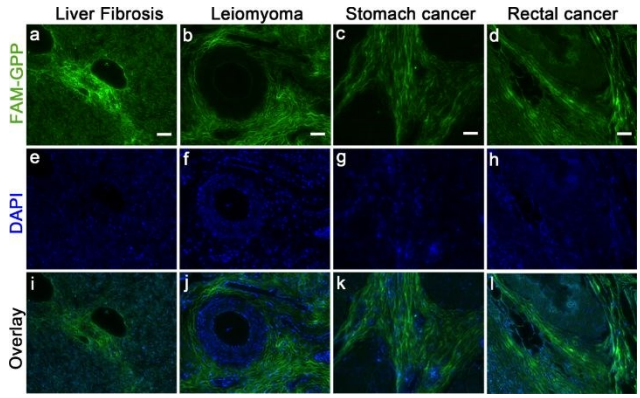


Figure 5. Pathological human tissue staining using unheated FAM-GPP. Fluorescence micrographs of liver fibrosis (a, e, i), leiomyoma (b, f, j), stomach cancer (c, g, k) and rectal cancer (d, h, l) tissue sections stained with preheated FAM-GPP (in green) and DAPI (in blue). Scale bar = 100 μm.

Similarly, fluorescence micrographs of FFPE tissues of human liver fibrosis, leiomyoma, stomach cancer, rectal cancer, esophageal cancer, breast cancer, liver cancer and lung cancer stained with unheated FAM-GPP and DAPI indicated that FAM-GPP could detect denatured collagen with high sensitivity and specificity in different types of connective tissues associated with cancers and other diseases (Figure 5, S9). These results suggested that the two peptide probes FAM-GOO and FAM-GPP possessed the capability to bind multiple types of collagen (e.g. type I in fibrosis and type IV in cancer) as well as collagen from different species (e.g. rat and human). Compared with antibodies specific for a certain type of collagen, both FAM-GOO and FAM-GPP are broad-spectrum probes for selectively detecting denatured collagen. Furthermore, both peptide probes could stain FFPE tissue sections without the need of antigen-retrieval, a critical step for antibody-staining.

Conclusions

Denatured collagen is a pivotal biomarker for a variety of pathological conditions such as cancer and fibrosis. Peptide probes with the repetitive $(Gly\text{-}Pro\text{-}Hyp)_n$ sequences have been recently found to target denatured collagen when they are in the monomer state.[37] However, the Gly-Pro-Hyp sequence is well-known as the most stabilizing triplet for triple helical structure, and the requirement of thermal or UV pretreatment to dissociate the peptides into the single stranded form results in severe drawbacks such as difficulty in accurate quantitation of effective pure monomeric probe and potential tissue damage. Therefore, there is an urgent need to create novel peptide probes that can target collagen and that has less triple helix propensity.

We herein have constructed two peptide probes FAM-GOO and FAM-GPP by the replacement of Pro and Hyp in the control peptide FAM-GPO with the well-characterized $(Gly\text{-}Pro\text{-}Hyp)_8$ sequence. Our CD, fluorescent and colorimetric studies have all demonstrated that FAM-GOO possessed similarly strong capability to form triple helix structure as FAM-GPO, while FAM-GPP showed a pronouncedly decreased triple helical stability, and maintained the monomer conformation at temperatures as low as 4 °C. The binding experiments have indicated that both peptide probes could recognize denatured collagen with high specificity, and thermal pretreatment was needed for FAM-GOO but not FAM-GPP. We have further shown that preheated FAM-GOO and unheated FAM-GPP could target denatured collagen in a broad variety of rat connective tissues, and the inhibitor experiments suggested the specific recognition was achieved via hybridization with the unfolded chains in denatured collagen.

Furthermore, preheated FAM-GOO and unheated FAM-GPP could detect denatured collagen with high sensitivity and specificity in different types of human pathological tissues. Compared with antibodies specific for a certain type of collagen, both FAM-GOO and FAM-GPP are broad-spectrum probes for selectively detecting denatured collagen of different types and from different species. Notably, peptide probe FAM-GPP always maintained the single stranded conformation by itself, without any need of thermal or UV pretreatment. This novel peptide probe composed of natural amino acids provides a convenient, biocompatible and highly specific biosensor for denatured collagen, which has great potential in the diagnosis and therapeutics of collagen-involved diseases.

Conflicts of interest

There are no conflicts to declare.

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References

1. A. Ferreira, I. Alho, S. Casimiro and L. Costa, *Bonekey Rep*, 2015, **4**, 668.
2. M. A. Karsdal, K. Henriksen, D. J. Leeming, P. Mitchell, K. Duffin, N. Barascuk, L. Klickstein, P. Aggarwal, O. Nemirovskiy, I. Byrjalsen, P. Qvist, A. C. Bay-Jensen, E. B. Dam, S. H. Madsen and C. Christiansen, *Biomarkers*, 2009, **14**, 181-202.
3. D. L. Motola, P. Caravan, R. T. Chung and B. C. Fuchs, *Curr Pathobiol Rep*, 2014, **2**, 245-256.
4. J. C. Rousseau and P. D. Delmas, *Nat Clin Pract Rheumatol*, 2007, **3**, 346-356.
5. C. E. Brinckerhoff, J. L. Rutter and U. Benbow, *Clin Cancer Res*, 2000, **6**, 4823-4830.
6. P. Caravan, B. Das, S. Dumas, F. H. Epstein, P. A. Helm, V. Jacques, S. Koerner, A. Kolodziej, L. Shen, W. C. Sun and Z. Zhang, *Angew Chem Int Ed Engl*, 2007, **46**, 8171-8173.
7. B. C. Fuchs, H. Wang, Y. Yang, L. Wei, M. Polasek, D. T. Schuhle, G. Y. Lauwers, A. Parkar, A. J. Sinskey, K. K. Tanabe and P. Caravan, *J Hepatol*, 2013, **59**, 992-998.
8. K. Gelse, E. Poschl and T. Aigner, *Adv Drug Deliv Rev*, 2003, **55**, 1531-1546.
9. R. Khan and R. Sheppard, *Immunology*, 2006, **118**, 10-24.
10. S. Ricard-Blum, *Cold Spring Harb Perspect Biol*, 2011, **3**, a004978.
11. R. C. Benyon and M. J. Arthur, *Semin Liver Dis*, 2001, **21**, 373-384.
12. D. H. Pashley, F. R. Tay, C. Yiu, M. Hashimoto, L. Breschi, R. M. Carvalho and S. Ito, *J Dent Res*, 2004, **83**, 216-221.
13. L. Troeberg and H. Nagase, *Biochim Biophys Acta*, 2012, **1824**, 133-145.
14. H. Morgan and P. A. Hill, *Cancer Cell Int*, 2005, **5**, 1.
15. V. M. Dejica, J. S. Mort, S. Laverty, M. D. Percival, J. Antoniou, D. J. Zukor and A. R. Poole, *Am J Pathol*, 2008, **173**, 161-169.
16. O. V. Nemirovskiy, D. R. Dufield, T. Sunyer, P. Aggarwal, D. J. Welsch and W. R. Mathews, *Anal Biochem*, 2007, **361**, 93-101.
17. Y. Aida, H. Abe, Y. Tomita, T. Nagano, N. Seki, T. Sugita, M. Itagaki, H. Ishiguro, S. Sutoh and Y. Aizawa, *J Gastrointestin Liver Dis*, 2015, **24**, 61-68.
18. X. Cheng, Y. Yang, Z. Fan, L. Yu, H. Bai, B. Zhou, X. Wu, H. Xu, M. Fang, A. Shen, Q. Chen and Y. Xu, *Oncogene*, 2015, **34**, 5570-5581.
19. T. R. Cox and J. T. Erler, *Dis Model Mech*, 2011, **4**, 165-178.

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DOI: 10.1039/D0TB01691H

20. G. Fredman, N. Kamaly, S. Spolitu, J. Milton, D. Ghorpade, R. Chiasson, G. Kuriakose, M. Perretti, O. Farokzhad and I. Tabas, *Sci Transl Med*, 2015, **7**.
21. J. Takagi, H. Asai and Y. Saito, *Biochemistry*, 1992, **31**, 8530-8534.
22. S. Federico, B. F. Pierce, S. Piluso, C. Wischke, A. Lendlein and A. T. Neffe, *Angew Chem Int Ed Engl*, 2015, **54**, 10980-10984.
23. H. Liang, X. Li, B. Chen, B. Wang, Y. Zhao, Y. Zhuang, H. Shen, Z. Zhang and J. Dai, *J Control Release*, 2015, **209**, 101-109.
24. W. Zhao, B. Chen, X. Li, H. Lin, W. Sun, Y. Zhao, B. Wang, Y. Zhao, Q. Han and J. Dai, *J Biomed Mater Res A*, 2007, **82**, 630-636.
25. J. M. Chan, L. Zhang, R. Tong, D. Ghosh, W. Gao, G. Liao, K. P. Yuet, D. Gray, J. W. Rhee, J. Cheng, G. Golomb, P. Libby, R. Langer and O. C. Farokhzad, *Proc Natl Acad Sci U S A*, 2010, **107**, 2213-2218.
26. L. M. De Leon-Rodriguez and Z. Kovacs, *Bioconjug Chem*, 2008, **19**, 391-402.
27. Y. Geng, P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko and D. E. Discher, *Nat Nanotechnol*, 2007, **2**, 249-255.
28. H. Y. Hu, N. H. Lim, D. Ding-Pfennigdorff, J. Saas, K. U. Wendt, O. Ritzeler, H. Nagase, O. Plettenburg, C. Schultz and M. Nazare, *Bioconjug Chem*, 2015, **26**, 383-388.
29. T. J. Moyer, H. A. Kassam, E. S. Bahnson, C. E. Morgan, F. Tantakitti, T. L. Chew, M. R. Kibbe and S. I. Stupp, *Small*, 2015, **11**, 2750-2755.
30. D. A. Rothenfluh, H. Bermudez, C. P. O'Neil and J. A. Hubbell, *Nat Mater*, 2008, **7**, 248-254.
31. B. H. San, J. Hwang, S. Sampath, Y. Li, L. L. Bennink and S. M. Yu, *J Am Chem Soc*, 2017, **139**, 16640-16649.
32. L. A. Liotta and E. C. Kohn, *Nature*, 2001, **411**, 375-379.
33. R. F. Loeser, S. R. Goldring, C. R. Scanzello and M. B. Goldring, *Arthritis Rheum*, 2012, **64**, 1697-1707.
34. M. McClung, S. T. Harris, P. D. Miller, D. C. Bauer, K. S. Davison, L. Dian, D. A. Hanley, D. L. Kendler, C. K. Yuen and E. M. Lewiecki, *Am J Med*, 2013, **126**, 13-20.
35. T. D. Tlsty and L. M. Coussens, *Annu Rev Pathol*, 2006, **1**, 119-150.
36. A. Y. Wang, X. Mo, C. S. Chen and S. M. Yu, *J Am Chem Soc*, 2005, **127**, 4130-4131.
37. Y. Li, C. A. Foss, D. D. Summerfield, J. J. Doyle, C. M. Torok, H. C. Dietz, M. G. Pomper and S. M. Yu, *Proc Natl Acad Sci U S A*, 2012, **109**, 14767-14772.
38. X. Cai, Z. Liu, S. Zhao, C. Song, S. Dong and J. Xiao, *Chem Commun*, 2017, **53**, 11905-11908.
39. M. D. Shoulders and R. T. Raines, *Annu Rev Biochem*, 2009, **78**, 929-958.
40. X. Sun, J. Fan, X. Li, S. Zhang, X. Liu and J. Xiao, *Chem Commun*, 2016, **52**, 3107-3110.
41. S. M. Yu, Y. Li and D. Kim, *Soft Matter*, 2011, **7**, 7927-7938.

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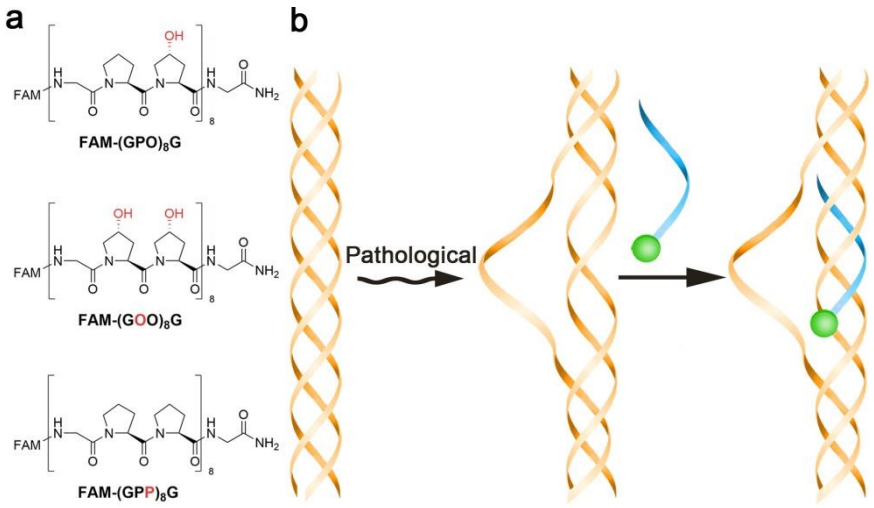
DOI: 10.1039/D0TB01691H



Two novel peptide probes provide versatile tools for specifically targeting denatured collagen in various types of connective tissues, which has attractive potential in the diagnosis and therapeutics of collagen-involved diseases.