Supplementary Information

A robust collagen-targeting MRI peptide contrast agent for in vivo imaging of hepatic fibrosis

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Materials and methods

Materials. Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-6-aminocaproic acid, Rink amide AM resin (200-400 mesh, loading = 0.345 mmol/g), hydroxy benzotriazole (HOBt) and O-(Benzotriazol-1-yl)-N, N, N', N'- tetramethyluronium hexafluorophosphate (HBTU) were purchased from GL Biochemical Company (Shanghai, China). 5(6)-carboxyfluorescein and $GdCl_3 \cdot 6H_2O$ were provided by Aladdin Industries (Shanghai, China). N, N-Diisopropylethylamine (DIEA), Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), tert-Butyl bromoacetate and benzyl bromoacetate were purchased from J&K Chemical Ltd. (Shanghai, China). H&E Stain Kit and goat serum were obtained from Solarbio Science & Technology Co. Ltd (Beijing, China). Cycle • 4HCl, tert-butyl bromoacetate, and benzyl 2-bromoacetate were purchased from Beijing InnoChem Science & Technology Co., Ltd (Beijing, China). Pentobarbital sodium was purchased from Kehao Biological Co., Ltd (Wuhan, China). Gadobutrol was obtained from Bayer Co. Ltd (Germany). Collagen Type I Rabbit Polyclonal antibody was purchased from Proteintech Group, Inc (Wuhan, China). All the commercial reagents were of analytical grade and were used without further purification.

Synthesis of the DOTA(tBu)$_3$. Cyclen·4HCl (200 mg, 1.16 mmol) (A) was initially dissolved in MeCN (200 mL). Sodium hydroxide (371 mg, 9.28 mmol, 8.0 eq.) was

added, and the resulting white suspension was stirred for 15 min under a nitrogen atmosphere. A solution of tert-butyl bromoacetate (660 mg, 3.40 mmol, 3.0 eq.) was slowly added to the above MeCN (50 mL) for more than 15 min, and the suspension was stirred for an additional 30 min at 25 °C before being refluxed for 16 hrs at 80 °C. The solvent was removed under vacuum and the residue underwent silica gel column chromatography (MeOH/DCM = 40/1 to 20/1 as eluent) to yield Tri-tert-butyl 2,2',2''-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate (C).

Compound C was dissolved in DMF, to which benzyl 2-bromoacetate and Na_2CO_3 were added, and the mixture was stirred at 80 °C. Upon completion of the reaction, a white precipitate was observed and subsequently separated by filtration. The filtrate was then recrystallized from a minimal volume of boiling toluene, and the crystallized product was washed with cold diethyl ether to yield tri-tert-butyl 2,2',2''-(10-(2-(benzyloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate (D).

A mixture of D (53.1 mg) and Pd/C (20.1 mg, 0.5 μmol) in MeOH (1 mL) was stirred in a test tube equipped with an H_2 balloon at 25 °C. The reaction was monitored continuously using thin-layer chromatography. After 12 hours, the mixture was filtered and washed with diethyl ether to remove the catalyst. The filtrates were combined and concentrated under vacuum. The resulting product was purified by silica gel column chromatography using a MeOH/DCM eluent gradient ranging from 20/1 to 10/1. The final product, DOTA(tBu)$_3$, was characterized by mass spectrometry and ^{1}H NMR. m/z calculated 573.7 $[M+H]^+$ for DOTA(tBu)$_3$, found 573.5 $[M+H]^+$ (Fig. S2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66-1.95 (m, 24H), δ 1.47-1.45 (m, 27H) (Fig. S3).

Synthesis of fluorescent peptide probe. Fluorescent peptide probe FAM-Ahx-LRELHLNNNG (F-ICTP) was synthesized in-house by standard Fmoc solid phase peptide synthesis method using Rink amide AM resin (0.345 mmol/g loading). Stepwise couplings of amino acids were performed using Fmoc-amino acids (4 eq.), HOBt (4 eq.), HBTU (4 eq.), and DIEA (6 eq.). The resin was thoroughly washed with DMF (3×10 mL) and DCM (2×10 mL) after each coupling step, and

the Fmoc protecting group was removed with 20% (v/v) piperidine in DMF. The Chloranil test was used to check the coupling reaction and Fmoc deprotection status. Once the synthesis of the peptide sequence was complete, the mixture of fluorophore FAM (10 eq.), HOBt (10 eq.), HBTU (10 eq.), and DIEA (16 eq.) in DMF was added to the resin and incubated for 24 hrs at 37 °C to conjugate FAM with the N-terminal of the peptide. TFA/H_2O (95:5) was applied to treat the resin for 2.5 hrs to deprotect the side-chain protecting group and remove the peptide from the resin. The peptide was harvested by precipitation with cold Et_2O. Crude products were collected by re-suspension of the peptide in cold Et_2O, sonication, and centrifugation. The peptide F-ICTP was purified and analyzed using reverse-phase HPLC on a C18 column. A mixture of water (0.1% TFA) and acetonitrile (0.1% TFA) was used as the mobile phase, with a linear gradient from 5% to 60% acetonitrile over 20 minutes. The purity of F-ICTP was determined to be 94.28% (Fig. S6a). The identity of the peptide was confirmed by mass spectrometry. m/z calculated 1650.6 $[M+H]^+$ for F-ICTP, found 1650.2 $[M+H]^+$ (Fig. S6b).

Synthesis of MRI peptide contrast agent. The peptide DOTA-Ahx-LRELHLNNNG (DOTA-Ahx-ICTP) was synthesized using the Fmoc solid phase peptide synthesis method with Rink amide AM resin and stepwise couplings of amino acids using Fmoc-amino acids (4 eq.), HOBt (4 eq.), HBTU (4 eq.), and DIEA (6 eq.). After each coupling step, the resin was washed with DMF and DCM, and the Fmoc protecting group was removed using 20% (v/v) piperidine in DMF. The Chloranil test was used to confirm successful coupling and Fmoc deprotection. After completing the synthesis of the peptide sequence, the mixture of DOTA $(tBu)_3$ (10 eq.), HOBt (10 eq.), HBTU (10 eq.), and DIEA (16 eq.) in DMF was added to the resin and incubated for 24 hrs at 37 °C to conjugate DOTA $(tBu)_3$ with the N-terminal of the peptide. TFA/TIS/H_2O (95:2.5:2.5) was applied to treat the resin for 2.5 hrs to deprotect the side-chain protecting group and remove the peptide from the resin. The purified peptide DOTA-Ahx-ICTP was dissolved in DI water and complexed with $GdCl_3 \cdot 6H_2O$ at 25 °C overnight to obtain the MRI peptide contrast agent Gd-DOTA-Ahx-ICTP (Gd-ICTP). The peptides DOTA-Ahx-ICTP and Gd-DOTA-Ahx-ICTP were purified using reverse-phase HPLC

on a C18 column. The purification process utilized a mobile phase consisting of water (0.1% TFA) and acetonitrile (0.1% TFA), with a linear gradient from 5% to 50% acetonitrile over 20 minutes. The purity of DOTA-Ahx-ICTP was determined to be 91.45%, while the purity of Gd-ICTP was found to be 98.70% (Fig. S4a, 5a). The identity of the peptides was confirmed by mass spectrometry. m/z calculated 1678.4 $[M+H]^+$ for DOTA-Ahx-ICTP, found 1678.0 $[M+H]^+$ (Fig. S4b). m/z calculated 1832.6 $[M+H]^+$ for Gd-ICTP, found 1831.7 $[M+H]^+$ (Fig. S5b). The xylenol orange colorimetric method was employed to measure free gadolinium ions. Relaxation times of the solution of Gd-ICTP with different concentrations were measured at 3.0 T Siemens MRI scanner in pH 7.4 phosphate-buffered saline (PBS) at 20 °C.

Protein binding assay. The binding assay of type I collagen, BSA, pepsin, trypsin, and silk protein hydrolysates followed a standard protocol. Solutions of each protein with a concentration of 100 μg/mL were prepared in 10 mM PB (pH 7.4). The protein solutions were added into the wells of a 96-well plate, and incubated at 4 °C for 5 hrs. The wells were washed with 300 μL 10 mM PBS (pH 7.4) for 3 min three times. 100 μL solution of BSA in 10 mM PBS (pH 7.4) (0.5% v/v) was added and incubated at room temperature for 1 hr to block non-specific binding. The wells were washed with 300 μL 10 mM PBS (pH 7.4) for 3 min three times. 50 μL solution of 15 nM peptide probe F-ICTP was added to each well, respectively, and incubated at 4 °C for 5 hrs to allow binding. The wells were washed with 300 μL 10 mM PBS (pH 7.4) for 3 min three times. Fluorescence (ex: 485 nm, em: 535 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each binding experiment was repeated three times.

Type I collagen (10 μg/mL) was prepared in 10 mM PBS (pH 7.4). 100 uL of type I collagen was added into each well of a 96-well plate, and air-dried. After coating, the type I collagen film was washed with 300 μL 10 mM PBS (pH 7.4) for 3 min three times. 100 μL solution of BSA in 10 mM PBS (pH 7.4) (0.5% v/v) was added and incubated at room temperature for 1 hr to block non-specific binding. 100 μL solution of 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 2 μM, 3 μM, 4 μM, 5 μM, 6 μM, and 10 μM peptide probe F-ICTP 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 300 µL 10 mM PBS (pH 7.4) for 5 min three times. Fluorescence (ex: 485 nm, em: 535 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). The K_d value was calculated by fitting the equilibrium dissociation constant (K_d) equation using GraphPad Prism 9 software.

Tissue staining and imaging. The animal experiments were carried out with the approval of the ethics committee of Lanzhou University No.1 Hospital, in compliance with appropriate ethical guidelines and regulations. Hepatic fibrosis was induced in KM mice (n = 60) by treatment with 1:2 CCl_4:olive oil mixture (70 µL/100 g), administered using intraperitoneal injection twice per week for 6 weeks. Control animals were treated with olive oil (n = 20). The liver tissues were obtained from these mice and cryopreserved in Tissue-Tek O.C.T. medium, sectioned to 4 µm thickness on glass slides, and air-dried at 25 °C for further analysis.

Tissue sections were added with 10% goat serum (10 mM PBS, pH 7.4) and incubated for 15 min at 25 °C to block nonspecific binding. F-ICTP solution was prepared at a concentration of 15 µM in 10 mM PB (pH 7.4), while the anti-collagen I antibody was diluted 500 times with 10 mM PB (pH 7.4). The tissue slides were coated with 100 µL of the peptide solution and incubated at 4 °C for 4 hrs, covered with parafilm. After removing the parafilm, the solutions on the slides were absorbed using bibulous paper. The tissue slides were then washed with 10 mM PB for 5 min, repeated three times. The anti-quenching agent was added to the tissue slides, which were covered with a cap. The stained tissue sections were then visualized using a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany).

MRI scan: All mice were imaged with a 3.0 T Siemens MRI scanner at the Lanzhou University No.1 Hospital. The MR scanning parameters used in the study were as follows: a Siemens 3.0T Skyra scanner equipped with a 5 cm aperture 4-channel coil. For T_1 scanning, the parameters were set as follows: TR (Repetition Time) of 500 mS, TE (Echo Time) of 8.6 mS, FOV (Field of View) of 100 mm, slice thickness of 1.5 mm, FOV phase at 100%, Dist (Distortion) factor at 20%, six averages, and

a total scan time of 7.3 min. Animals were anesthetized and the temperature was maintained with a small animal physiological monitoring system. T_1-weighted MRI images were scanned prior to and at 2 hrs after intravenous administration of 7.5 µmol/kg of Gd-ICTP in healthy and fibrosis mice. Additionally, T_1-weighted MRI images of fibrosis mice injected with the same dose of gadobutrol were acquired prior to and at 2 hrs after intravenous administration.

Statistical analysis: Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). The contrast-to-noise ratio (CNR) in the liver was calculated by selecting a region excluding any large blood vessels, estimating the mean intensity, and then subtracting it from the mean intensity of muscle. This value was then divided by the standard deviation of a region in the air adjacent to the animal. The change in CNR was calculated as a difference: $\Delta CNR = (CNR_{2h}-CNR_{0h})$ where "CNR_{2h}" refers to the CNR at 2 hrs after probe injection and "CNR_{0h}" refers to the CNR of the image taken before probe injection. The p-values were calculated using an unpaired two-tailed Student t-test.

Histopathological analysis and biodistribution. The mice used in the MRI study were sacrificed by cervical dislocation 2 hrs post-injection. Subsequently, the samples were fixed in formalin, embedded in paraffin, sectioned into 4 µm slices, and subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining following established protocols. The organs, including the lung, kidney, heart, and liver, were immediately removed, rinsed in saline, and then blotted dry. Tissue samples were digested overnight in concentrated nitric acid. The resulting HNO_3 solution containing the digested tissues was collected the following day, filtered, and diluted with 2% (wt/vol) HNO_3. Then each sample from different organs was analyzed by ICP-OES to measure the gadolinium ion concentration. The Gd content was expressed as the percentage of injected dose per gram of tissues (% ID Gd/g tissue).

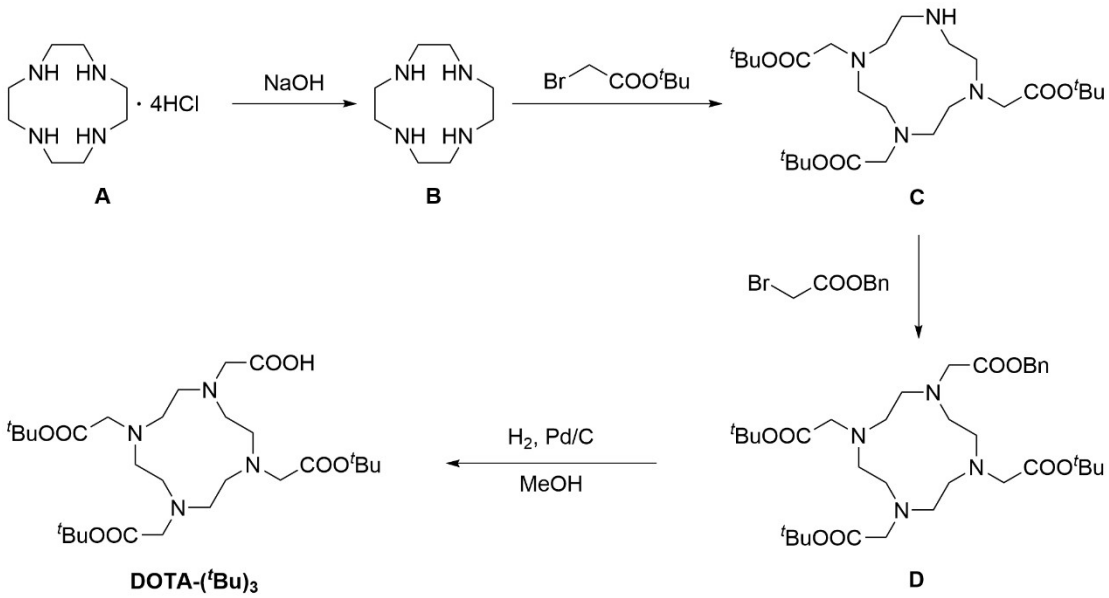

Fig. S1. Schematic diagram of the synthesis of DOTA(tBu)$_3$.

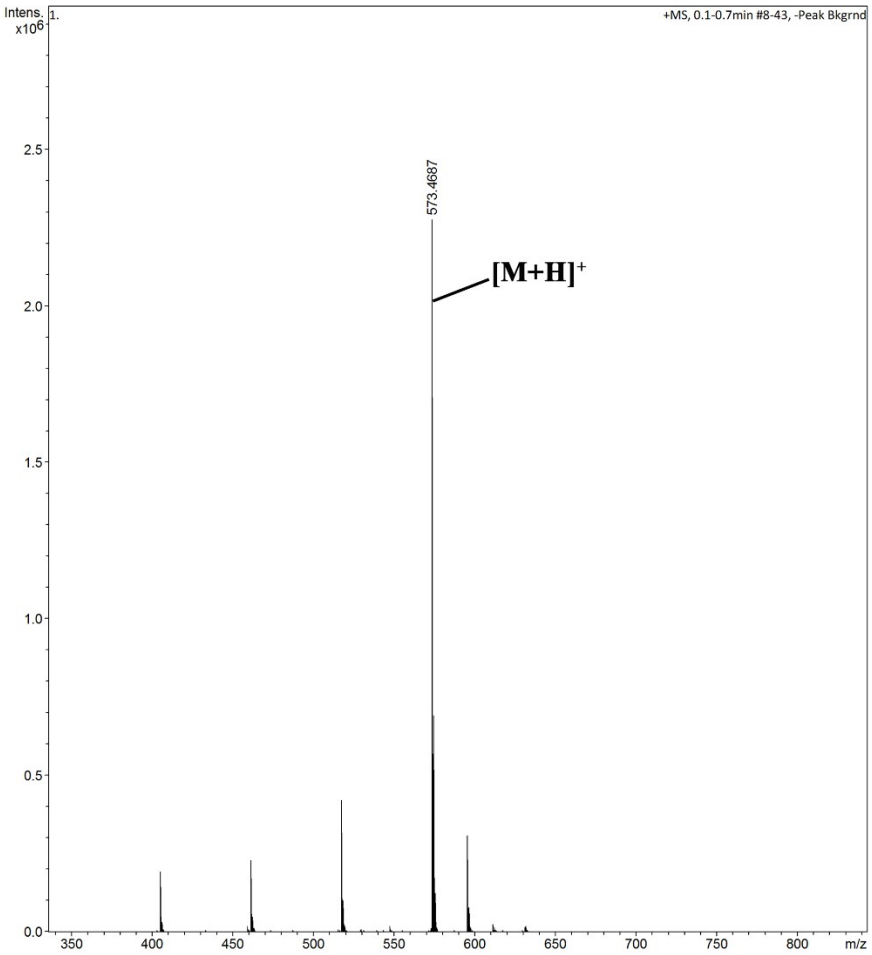

Fig. S2. Mass spectrum of DOTA(tBu)$_3$.

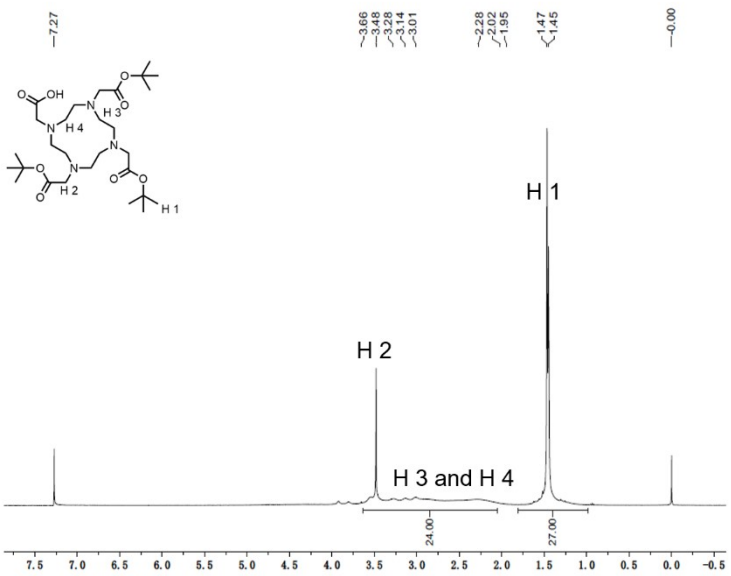

Fig. S3. ^{1}H NMR spectrum of DOTA(tBu)$_3$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.66-1.95 (m, 24H), δ 1.47-1.45 (m, 27H).

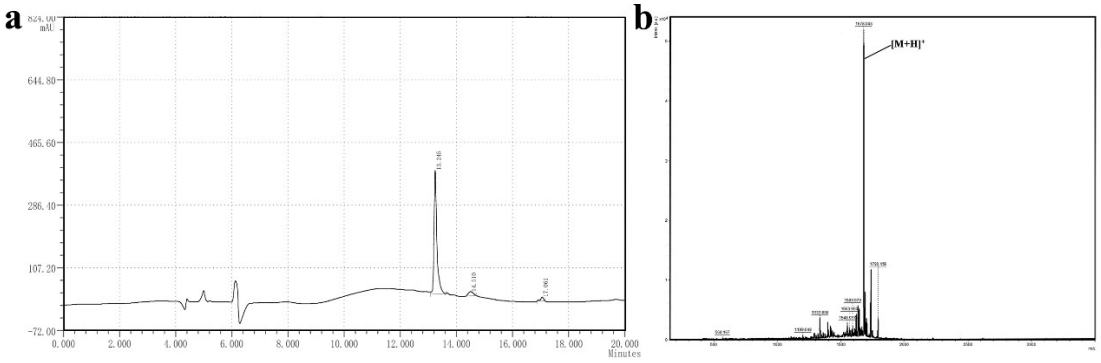

Fig. S4. HPLC chromatogram (a) and Mass spectrum (b) of DOTA-Ahx-ICTP. m/z calculated 1678.4 $[M+H]^+$ for DOTA-Ahx-ICTP, found 1678.0 $[M+H]^+$; t_R = 13.2 min.

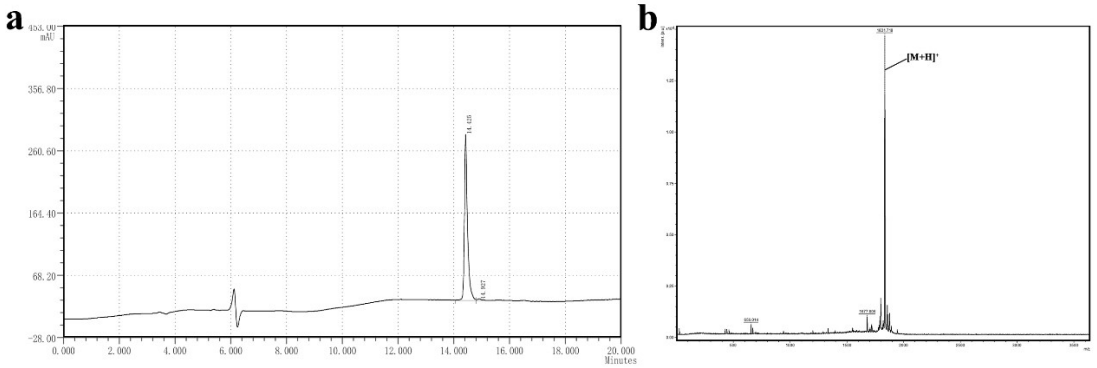

Fig. S5. HPLC chromatogram (a) and Mass spectrum (b) of Gd-ICTP (Gd-DOTA-Ahx-ICTP). m/z calculated 1832.6 $[M+H]^+$ for Gd-ICTP, found 1831.7 $[M+H]^+$; t_R = 14.4 min.

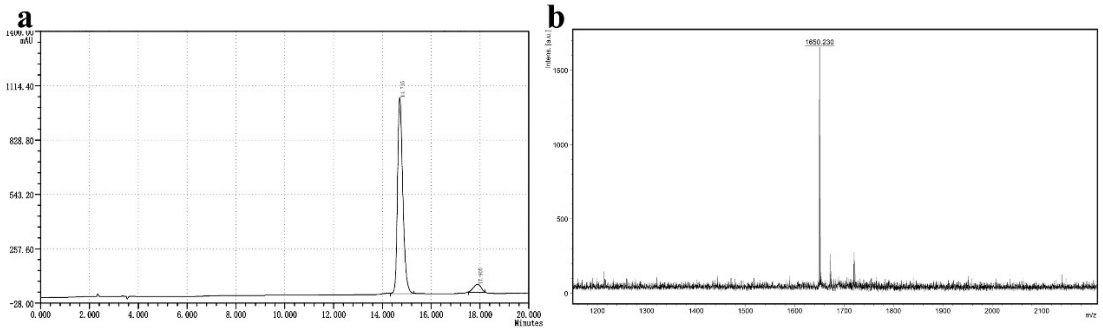

Fig. S6. HPLC chromatogram (a) and Mass spectrum (b) of F-ICTP. m/z calculated 1650.6 $[M+H]^+$ for F-ICTP, found 1650.2 $[M+H]^+$. t_R = 14.7 min.

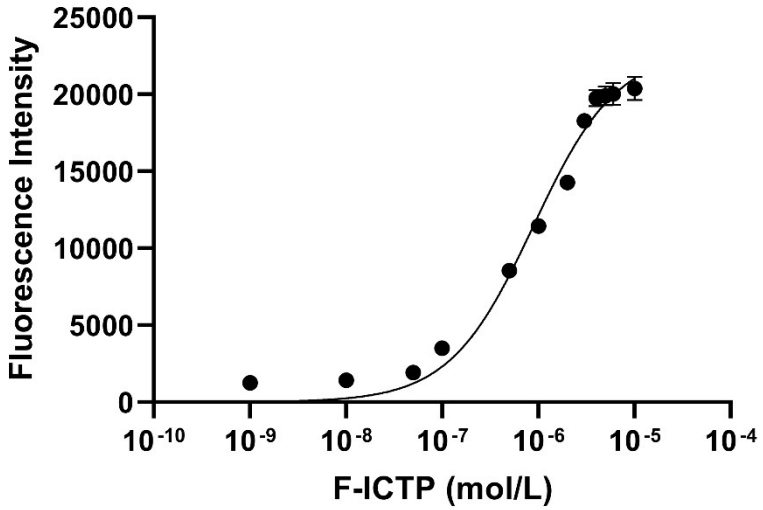

Fig. S7. The binding affinity of F-ICTP with type I collagen.