Supporting Information to:

A fluorogenic monolayer to detect the co-immobilization of peptides that combine cartilage targeting and regeneration

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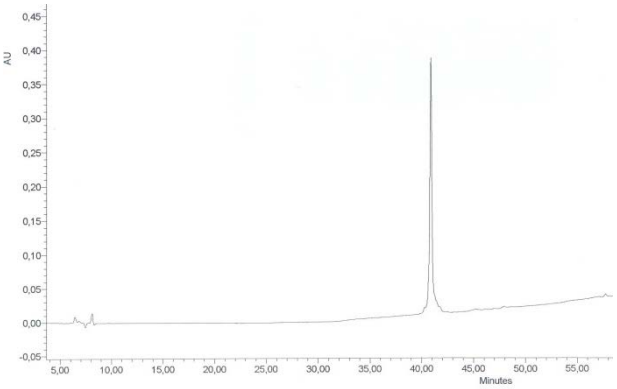
Materials

Commercial grade reagents were purchased from Sigma-Aldrich and were used without further purification unless stated otherwise and solvents were dried over molecular sieves. The control scrambled peptide sequences were adapted from the previous references or established by randomly scrambling its aminoacids and confronting the resulting sequences with literature studies reporting biological activity (or lack thereof) for a given sequence. Recombinant human TGF-β1 was purchased from R&D Systems as a 2 µg carrier-free lyophilized powder from a 0.2 µm filtered solution in acetonitrile and TFA. TGF-β1 was reconstituted according to the protocol of the provider at a concentration of 20 µg/mL and further diluted to the desired working concentration in PBS buffer. Collagen type II from bovine nasal septum was purchased as a powder and reconstituted as indicated by the provider to a stock concentration of 5 mg/mL and further diluted to the desired working concentration in PBS buffer. High-purity water (Millipore, R=18.2 MΩcm) was used. Electrospray Ionization (positive mode) high resolution mass spectra were recorded on a WATERS LCT mass spectrometer. For information on the synthesis and characterization of the compounds **1** and **2** the reader is referred to the work of Nicosia et al.

Automated solid-phase peptide synthesis

Fmoc-Rink amide MBHA resin (Multisyntech GmbH, 50 mg, substitution 0.64 mmol/g) was placed in the reaction vessel of the fully-automated peptide synthetizer (Syro), and swollen for 30 min in 3 mL N-methyl-2-pyrrolidone (NMP) (Biosolve BV). After emptying the vessels the resin was washed with 1 mL piperidine (40%v)/NMP for 3 min and with 1 mL piperidine (20%v)/NMP for 12 min. The resin was washed with NMP (6 x 1mL) prior to adding the following solutions to the vessel: Fmoc-amino acid (Multisyntech GmbH, 4.5 equivalents with respect to the resin) dissolved in a solution of HOBT (Biosolve BV, 0.3 M) in NMP, HBTU (Multisyntech, 0.29 M) in NMP and DIPEA (0.58 M) in NMP. The solid phase reaction was carried out for 80 min at room temperature and the vessel was emptied under reduced pressure. The deprotection and coupling cycles were repeated until the synthesis of the Fmoc protected peptide sequence was complete. The cleavage of the side chain protecting groups and the release of the sequence from the resin was achieved by manually incubating the resin with a mixture of 95% trifluoroacetic acid (TFA) (Acros), 2.5% H_2O, 2.5% 1,2-ethanedithiol (EDT) and 1% triisopropylsilane (TIS) overnight. The peptides were purified by several cycles of precipitation using cold diethyl ether (Acros), redissolved in water and lyophilized overnight. LC-MS (LCQ Fleet from Thermo Scientific on a C18 column using a gradient from 0 to 90% of B (solvent A = 0.1% FA/H_2O; solvent B = 0.1% FA/CH_3CN): m/z = 839.8 $[M+H]^+$, (calcd. 839.0 for $C_{35}H_{58}N_{12}O_{10}S$) for CLPLGNSH. LC-MS (ESI): m/z = 840.2 [M+H]+, (calcd. 839.0 for $C_{35}H_{58}N_{12}O_{10}S$) for CHNLGLPS. MS (ESI): m/z = 952.8 [M+H]+, (calcd. 952.1 for $C_{43}H_{65}N_{15}O_8S$) for CLRGRYW. MS (ESI): m/z = 952.4 [M+H]+, (calcd. 952.1 for $C_{43}H_{65}N_{15}O_8S$) for CWRGLRY.



Substrate and monolayer preparation

Microscope glass slides were used for monolayer preparation. [12-13] The substrates were oxidized with piranha solution for 30 min (concentrated H_2SO_4 and 33% aqueous H_2O_2 in a 3:1 ratio; (***Warning!*** *Piranha solution should be handled carefully: it has been reported to detonate unexpectedly*) and rinsed with water (MilliQ). After drying them in a flow of N_2, the substrates were immediately immersed in 0.1 vol.% 11-bromoundecyltrichlorosilane (ABCR) in dry toluene for 45 min at room temperature. Following monolayer formation, the substrates were rinsed with toluene to remove any excess of silanes, with ethanol and subsequently dried in a N_2 flow. Next, the bromo-functionalized substrates were immersed into a saturated solution of sodium azide (NaN_3) (Acros) in DMF for 48h at 70°C. The substrate were thoroughly rinsed with MilliQ water and ethanol and dried in a N_2 flow.

PDMS preparation and µCP experiments

Stamps were prepared by casting a 10:1 (v/v) mixture of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) against a silicon master. After overnight curing at 60°C, the stamps were oxidized by oxygen plasma for 10 sec (power tuned at 50 mA) and subsequently inked by dropping the inking solution onto the stamp (1.5 mM **1** (in CH_3CN), 0.5 mM Cu(I)$(CH_3CN)_4PF_6$ and 0.5 mM TBTA (CH_3CN/EtOH=2/1) (catalyst mixture). The inking solution was prepared by mixing 75 µL of 2 mM solution of **1** in CH_3CN and 25 µL of 2 mM of catalyst mixture). After inking the stamps for 4 min, the stamps were blown dry in a flow of N_2 and subsequently brought into conformal contact with the azide-functionalized substrate for 60 min. Fresh stamps were used for each printing following the same inking procedure. After removal of the stamp, the printed substrates were rinsed with ethanol, sonicated in acetonitrile for 2 min, rinsed again with ethanol, blown dry with N_2 and imaged using fluorescence microscopy. Printing was performed by inking PDMS for 30 min with a 10 mM solution of peptide in PBS. After removing the excess of the inking solution and drying the stamps in a flow of N_2, the stamps were brought into conformal contact with the substrate for 60 min.

Interfacial reaction with thiols

After immobilizing **1**, the platform was used for reacting with cysteine-terminated peptides from solution. Solutions of 10 mM of L-cysteine ethyl ester hydrochloride and peptides were prepared in PBS at pH 7.4 or in DMSO:PBS (3:1) and used for incubating the patterned substrates for 1h. Substrates were then rinsed and briefly sonicated in PBS and in MilliQ water. For cell adhesion experiments a backfilling with a hydrophilic compound was necessary to improve the selectivity of the cell patterning. Therefore after reaction with some samples were incubated in a water solution

containing 30 mM propargylhexa(ethylene glycol), [9] 50 mM NaCl, 1 mM $CuSO_4$ and 40 mM ascorbic acid over night at room temperature.

TGF-β1 and BCII binding and immunofluorescent staining

Upon functionalization of the surfaces with peptides, the substrates were incubated with solutions containing either 1 µg/mL TGF-β1 in PBS, 50 µg/mL BCII in PBS or both together for 1 h at 37 °C. The substrates were then rinsed three times 10 min with PBS buffer including 1 mM Tween-20 on the orbital shaker and finally rinsed with PBS buffer. Afterwards, substrates were incubated with PBS containing 1% BSA for 1 h at room temperature and washed again three times as described above to block the background against non-specific protein interactions. Protein patterns were then incubated at room temperature with 200 µL of a solution of 5 µg/mL of monoclonal anti-TGF-β1 antibody (R&D systems) produced in mouse (mouse IgG_{2b} isotype, clone 141322) in PBS buffer including 1% BSA. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N_2. Consecutively, 200 µL of a solution (4 µg/mL) of anti-mouse IgG_{2b} labeled with Alexa Fluor® 546 produced in goat (Invitrogen) in PBS buffer (including 1% BSA) were used to incubate the samples for 1 h at room temperature. The substrates were then washed again three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N_2 and used for fluorescence microscopy imaging. For staining collagen II, polyclonal anti-collagen II α1 produced in rabbit (Abnova) was diluted 100 times in PBS buffer (including 1% BSA) and 200 µL of this solution where used to incubate each patterned sample for 1 h at room temperature. Samples were washed as described above. Subsequently, a solution of 20 µL/mL of Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Life technologies™) in PBS (including 1% BSA) was used to incubate the substrates for 1 h at room temperature and washed and dried prior to inspection by fluorescence microscopy.

Cell differentiation studies

Macroscopically intact articular cartilage from femoral condyles was obtained from patients undergoing total knee replacement. Chondrocytes were isolated after overnight digestion with Collagenase type II and used at passage two.
Chondrocytes were plated at a density of 5000 cells/cm^2 in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich), supplemented with 4.5 g/L glucose, 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich), 50 µg/mL L-ascorbic acid-2-phosphate (AsAP), 100 µg/mL sodium pyruvate 40 µg/ml proline, 1% of a mixture of 1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin (substantially iron-free), and 0.5 µg/ml sodium selenite (ITS supplement) and 10^{-7} M dexamethasone. 100 ng of TGF-β1 was either supplemented to the medium or bound to the surfaces as indicated in the results and discussion section. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for 7 days.
Live/Dead assay (LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Molecular Probes®) was performed according to the protocol from the provider.

GAG quantification and staining

For the quantification of GAGs in cell lysates, HACs were digested in a proteinase K (1 mg/mL) digestion buffer at 56°C overnight. Briefly, the digestion buffer was freshly prepared by mixing 100 mL of Tris/EDTA buffer (6.055 g Tris and 0.372 g EDTA in 1 L water adjusting pH to 7.6 with HCl) with

18.5 g iodoacetamide. After warming this solution to 56°C for 1 h 1 mg pepstatin A and proteinase K were added to complete the digestion buffer. After digestion, GAG levels were quantified using 25 µL of each digested sample and adding 5 µL of 2.3 NaCl and 150 µL of dimethylmethylene blue (DMMB) solution (95 mL 0.1 M HCl, 2.04 g glycine, 2.37 g NaCl and 16 mg DMMB in 1 L water, adjusting pH to 3.0 using HCl). Immediately after adding the DMMB solution, the absorbance was recoreded both at 525 and 590 nm and their quotient was used to determine the GAG concentration according to a standard curve ranging from 0 to 100 µg/mL of GAGs. GAG levels were corrected for the DNA content, which were measured using the CyQuant Cell Proliferation assay (Invitrogen).
For GAG staining, cells were fixed in 10% buffered formalin and stained using 0.5% Alcian Blue (Sigma Aldrich) in H_2O (pH set to 1 using HCl) for 30 minutes.

Fluorescence microscopy

Fluorescence microscopy images were recorded using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition. UV excitation (300 nm ≤ λ_{ex} ≤ 400 nm) and blue emission (410 nm ≤ λ_{em} ≤ 510 nm) was filtered using a Dapi Olympus filter cube. Green excitation (510 nm ≤ λ_{ex} ≤ 550 nm) and red emission (λ_{em} ≥ 580) was filtered using a Olympus filter cube. All fluorescence microscopy images were acquired in air.

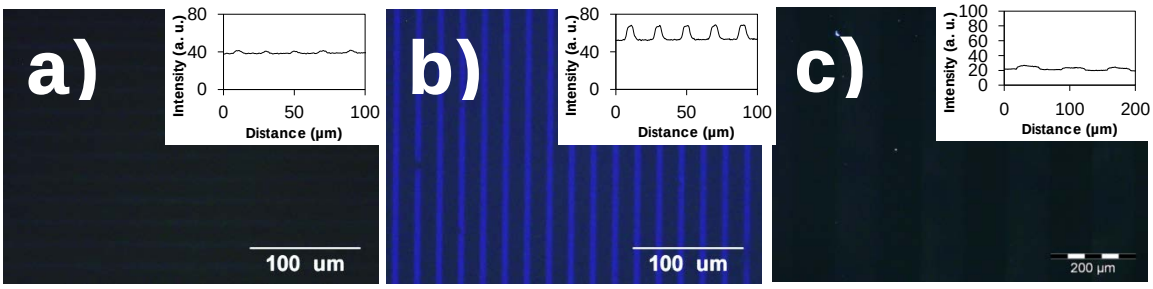


Figure S1: Fluorescence microscopy images corresponding to substrates patterned with a) coumarin **1** and after incubation with b) 10 mM L-cysteine ethyl ester hydrochloride and c) 10 mM of a methionine end-capped peptide **2** following Scheme 1. Insets show fluorescence intensity profiles.