# Assembling and decorating hyaluronan hydrogels with twin protein superglues to mimic cell-cell interactions

Robert Wieduwild and Mark Howarth*

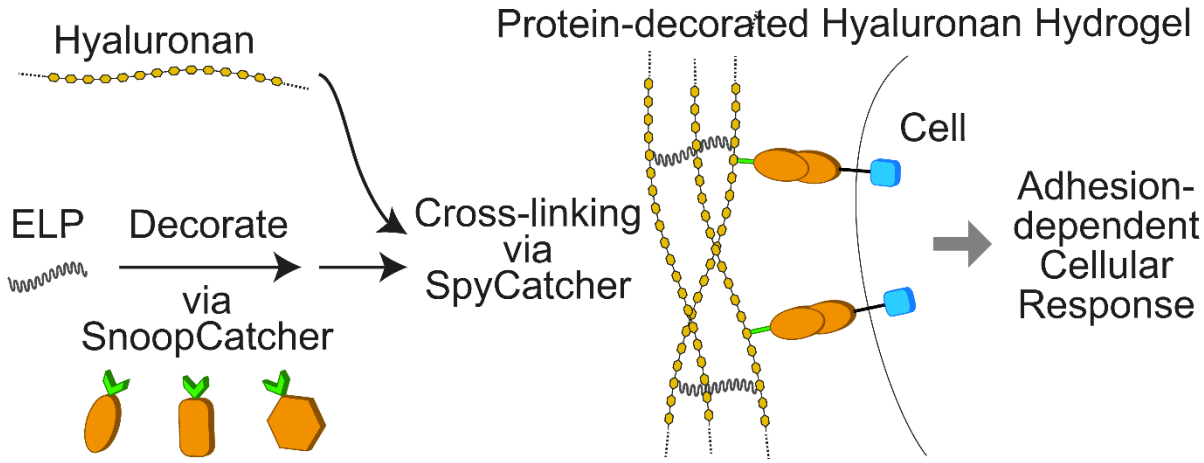
Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK

* E-mail: mark.howarth@bioch.ox.ac.uk

## Abstract

Simple polymeric scaffolds have yielded dramatic effects on cell behavior. For more sophisticated phenotypes, precise and efficient chemistries are desired to incorporate proteins into these scaffolds. Here we derivatize hyaluronan with an elastin-like polypeptide containing telechelic SpyTags (HA-SpyTag). Our second network component, the TriCatcher protein, had two SpyCatchers and a terminal SnoopCatcher. Mixing HA-SpyTag with TriCatcher led to rapid hydrogel formation, via spontaneous amidation. SnoopCatcher allowed modular network decoration with SnoopTagJr-linked adhesion molecules, through orthogonal transamidation. This programmed scaffold enables the testing of how individual matrix-anchored protein interactions affect cell behavior. Epithelial cell adhesion molecule (EpCAM) regulates cell behavior and migration, with important effects in cancer. EpCAM-anchoring to the hydrogel induced disassembly of non-malignant mammary spheres in 3D culture. Integrating signaling proteins into biomaterials via efficient biocompatible chemistry should reveal key cues to control cell behavior.

## Graphical Abstract




## Keywords

Protein engineering; bioconjugation; polysaccharide; hyaluronic acid; glycobiology; bioengineering.

## Introduction

Biomaterials have the potential to revolutionize drug delivery, tissue engineering and regenerative medicine.[1] Biomaterials have shown initial success in promoting the development of sophisticated multi-component tissues, including segments of skin and bone.[2, 3] To mimic native tissue, polymers must possess a precise combination of mechanical, structural and biochemical features.[4] Most work on biomaterials has focused on identifying suitable repetitive polymers able to drive cellular behavior.[1-3] Proteins are harder to couple specifically than peptides or repetitive polymers, but can provide valuable structural

and biochemical cues within biomaterials.[1, 5, 6] A major challenge is to specifically and covalently integrate proteins into biomaterials with high efficiency.

Diverse chemical or enzymatic strategies can link proteins to hydrogels.[7] However, challenges have included interference with protein folding (e.g. coupling through cysteine interfering with disulfide bonds), promiscuous ligation (transglutaminase), reversible reaction (sortase A) or undesired side-reactions (peroxidases).[7] Unnatural amino acid incorporation allows precise covalent bond formation, but with the challenge of increased cost and complexity for expression.[8] We previously reported a route to specific covalent bond formation, by engineering a collagen adhesion domain (CnaB2) from *Streptococcus pyogenes*.[9] By genetically splitting CnaB2, a peptide tag (SpyTag) and protein partner (SpyCatcher) were developed, rapidly forming an intermolecular isopeptide bond (Figure 1a). This system shows good specificity, does not undergo reversal or side-reactions, and is tolerant to a range of buffers and temperatures.[9, 10] SpyTag/SpyCatcher technology was used by the Arnold and Tirrell groups to design branched structures and hydrogels using elastin-like polypeptides (ELPs).[11] Published SpyTag/SpyCatcher-based hydrogels applied SpyTag for both network assembly and incorporation of bioactive factors.[11-14] In 2016 we published a non-cross-reacting (orthogonal) pair to SpyTag/SpyCatcher: the peptide SnoopTag forms a covalent bond with the protein partner SnoopCatcher through spontaneous transamidation (Figure 1a).[15] Here we take advantage of these orthogonal pairs to assemble a stable hydrogel, which can be functionalized with adhesion proteins to modulate mammalian cell behavior in 3D culture.

## Experimental part

**Cloning.** Q5 High-Fidelity 2× Master Mix (New England BioLabs) was used for all PCRs. Constructs were initially cloned into chemically competent *E. coli* NEB® turbo (New England BioLabs).

pET28a SnoopTagJr-MBP (maltose binding protein) (GenBank accession no. MG867374, Addgene plasmid ID 105628), pET28a SpyTag-MBP (GenBank accession no. KU356870.1, Addgene plasmid ID 35050) and pET28a SnoopCatcher (GenBank accession no. KU500646, Addgene plasmid ID 72322) have been described.[15, 16]

pQE80L TriCatcher-RGDSP (Figure S1, GenBank accession no. MH511519, Addgene plasmid ID 112631) was generated by Gibson isothermal assembly in two steps. ΔN1-SpyCatcher-helical linker-SnoopCatcher in pET28a was amplified using 5′-GATAGTGCTACCCATATTAAATTCTCAAAACGTGATGAGG and 5′-TTTGTTAGCAGCCGGATCCTTATTTCGGCGGTATCGGTTCATTGGTGATATAATGTTTAC and cloned into the pQE-BB backbone amplified using 5′-CCTCATCACGTTTTGAGAATTTAATATGGGTAGCACTATC and 5′-AACATTATATCACCAATGAACCGATACCGCCGAAATAAGGATCCGGCTGCTAACAAAGC C, complementing the ΔN1-SpyCatcher to a full length SpyCatcher (pQE80L SpyCatcher–SnoopCatcher).[15] pQE-BB (containing SpyCatcher-MMP-ELP-RGDSP-ELP-SpyCatcher-MMP) was kindly provided by David Tirrell, Caltech.[11] pQE80L TriCatcher-RGDSP was assembled using three fragments. Fragment 1 was amplified from pQE-BB using 5′-GGTAGCGGTGGCTCTGGCGGTCAGCTCGACGG and 5′-CCTCATCACGTTTTGAGAATTTAATATGGGTAGCACTATC, mutating the MMP-cleavable linker PQGIWGQ next to the N-terminal SpyCatcher to GSGGSGGQ at the same time. Fragment 2 was amplified from pQE80L SpyCatcher–SnoopCatcher using 5′-GATAGTGCTACCCATATTAAATTCTCAAAACGTGATGAGG and 5′-GCCTACATACCTCGCTCTGC. The last fragment complemented the final construct using 5′- GCAGAGCGAGGTATGTAGGC and 5′-CCAGAGCCACCGCTACCGTCAATATGAGCGTCACCTTTAGTTG, mutating the MMP-cleavable linker PQGIWGQ next to the N-terminal SpyCatcher to GSGGSGGQ at the same time.

pQE80L TriCatcher-ELP (Figure S1, GenBank accession no. MH511518, Addgene plasmid ID 112630) was generated by Gibson isothermal assembly. The central RGDSP of

pQE80L TriCatcher-RGDSP was mutated to a non-integrin binding RGRAP by creating two PCR fragments using 5′-GGTCGCGGTCGCGCCCCGGCCAGCTCTGCC and 5′-GCGCGACCGCGACCGGTAACCGCATAG, in combination with 5′-GCAGAGCGAGGTATGTAGGC and 5′-GCCTACATACCTCGCTCTGC binding the origin of the plasmid.

pQE80L TriCatcher-MMP (Figure S1, GenBank accession no. MH511520, Addgene plasmid ID 112632) was generated by Gibson isothermal assembly. The central sequence VTGRGDSPASSAPI of pQE80L TriCatcher-RGDSP was mutated to RGRAPAVPLSLYSGIR by creating two PCR fragments using 5′-CCGTTCCGCTCAGTCTCTATAGTGGTATCCGTGCCACTAGTGTGCCGGG and 5′-GAGACTGAGCGGAACGGCCGGACGACCACGCGCATAGAGCTCACCAACG, in combination with 5′-GCAGAGCGAGGTATGTAGGC and 5′-GCCTACATACCTCGCTCTGC binding in the origin of the plasmid. The RGDSP sequence was thereby mutated to the non-integrin binding sequence RGRAP, in combination with introducing the MMP-cleavable sequence VPLSLYSG, designed to be a good substrate for a range of different MMPs.[17]

pQE80L TriCatcher-RGDSP-MMP (Figure S1, GenBank accession no. MH511521, Addgene plasmid ID 112633) was generated by Gibson isothermal assembly. The central sequence VTGRGDSPASSAPI of pQE80L TriCatcher-RGDSP was mutated to RGDSPAVPLSLYSGIR using 5′-GAGACTGAGCGGAACGGCCGGACTATCACCACGCGCATAGAGCTCACCAACGCCCG and 5′-CCGTTCCGCTCAGTCTCTATAGTGGTATCCGTGCCACTAGTGTGCCGGG, in combination with 5′-GCAGAGCGAGGTATGTAGGC and 5′-GCCTACATACCTCGCTCTGC binding in the origin of the plasmid. The RGDSP sequence was kept and the same MMP-cleavable sequence VPLSLYSG introduced as in pQE80L TriCatcher-MMP.

pQE80L SpyTag-ELP-SpyTag (Figure S2, GenBank accession no. MH511522, Addgene plasmid ID 112634) was generated by Gibson isothermal assembly. pQE-AA (containing SpyTag-ELP-RGDSP-ELP-SpyTag) was kindly provided by David Tirrell, Caltech.[11] The central RGDSP of pQE-AA was mutated to the non-integrin binding RGRAP and the neighboring serine was mutated to a cysteine using 5′-CCGTGGTCGTGCCCCGGCCTGCTCTGCC and 5′-GGGCACGACCACGGCCGGTAACC, in combination with 5′-GCAGAGCGAGGTATGTAGGC and 5′-GCCTACATACCTCGCTCTGC binding in the origin of the plasmid.

pQE80L SpyTag-ELP-SpyTag DK (Figure S2, GenBank accession no. MH511523, Addgene plasmid ID 112635) was generated by Gibson isothermal assembly. The first fragment covered the sequence from the N-terminal to the C-terminal SpyTag, mutating the reactive aspartic acid of the C-terminal SpyTag to a lysine (SpyTag DK), using 5′-CCGACCAAACTCGACGGC and 5′-GCGTCGAGCAGCCCG. Fragment 2 spanned from the C-terminal SpyTag DK to the origin of the plasmid using 5′-CGGGCTGCTCGACGCCCATATTGTCATGGTTAAAGCATACAAGCCG and 5′-GCCTACATACCTCGCTCTGC. The last fragment complemented the final construct, starting at the origin of plasmid using 5′-GCAGAGCGAGGTATGTAGGC and 5′-GCCGTCGAGTTTGGTCGG spanning to the N-terminal SpyTag.

pENTR4 EpCAM-SnoopTagJr (Figure S2, GenBank accession no. MH511516) was generated by Gibson isothermal assembly. pGEM-EPCAM (#HG10694-G) was from Sino Biological. In the first step, three fragments were assembled. First the extracellular portion of human EpCAM (residue 24-265) from pGEM-EPCAM was amplified using 5′-CGCCCGGTTCCGGAGACAGGAAGAATGTGTCTGTGAAAACTACAAGCTG and 5′-ATCGCCCAGCTTCCCGGATCCTCCGCTGCCTTTTAGACCCTGCATTGAGAATTCAGGTGC. The second fragment ranged from the C-terminal insertion site of pENTR4−Pfs25−SpyTag to the origin of the plasmid, using 5′-GGATCCGGGAAGCTGGGCGATATTGAATTTATTAAGGTGAACAAGTAAGAATTCCTCGAGGCGGCCG and 5′-GCAGAGCGAGGTATGTAGGC.[18] The third fragment covered the sequence from the origin until the tissue plasminogen activator (tPA) secretion leader sequence using 5′-GCCTACATACCTCGCTCTGC and 5′-TCTCCGGAACCGGGCG. In the next step a plasmid containing EpCAM-SnoopTagJr was assembled using two fragments,

aiming to increase the distance between EpCAM and the SnoopTagJr (changing $GSG_2$ to $GSG_4$), additionally to inserting a C-terminal GS sequence, to improve the overall SnoopTagJr availability and reactivity. The first fragment covered SnoopTagJr to the origin of the newly created plasmid using 5′-GCTGGGCAGCATTGAATTTATTAAGGTGAACAAGGGCAGCTAAGAATTCCTCGAGGCGGC and 5′-GCAGAGCGAGGTATGTAGGC. The second fragment completed the plasmid by ranging from the origin to SnoopTagJr using 5′-GCCTACATACCTCGCTCTGC and 5′-TTAATAAATTCAATGctGCCCAGCTTTCCGCTCCCGCCGGATCCCCCGGATCCTCCGCTG. The final plasmid (changing the $GSG_4$ to $GSG_2$-$His_6$-$GSG_2$ for Ni-NTA purification) was assembled using two fragments with 5′-CACCATCACCATCACCATGGCAGCTAAGAATTCCTCGAGGC and 5′-GCAGAGCGAGGTATGTAGGC, amplifying from the GSG linker to the origin and using 5′-GCCTACATACCTCGCTCTGC and 5′-GCCATGGTGATGGTGATGGTGGCCGCTGCCCTTGTTCACCTTAATAAATTCAATGC amplifying from the origin to the GSG linker.

pENTR4 EpCAM (Figure S2, GenBank accession no. MH511515) was generated by Gibson isothermal assembly. pENTR4 EpCAM was created by removing the SnoopTagJr sequence from pENTR4 EpCAM-SnoopTagJr using 5′-GCCTCGAGGAATTCTTAGCTGCCATGGTGATGGTGATGGTGCCC and 5′-GCCTACATACCTCGCTCTGC to amplify $His_6$ with a stop codon until the origin and primers 5′-GCAGAGCGAGGTATGTAGGC and 5′-GGCAGCTAAGAATTCCTCGAGGC to amplify the origin until the $His_6$ with a stop codon.

pENTR4 E-cadherin-SnoopTagJr (Figure S2, GenBank accession no. MH511517) was generated by Gibson isothermal assembly, based on E-cadherin-GFP, a gift from Jennifer Stow (Addgene plasmid # 28009).[19]

Complete inserts for all constructs were confirmed by Sanger sequencing.

**Protein Expression in *E. coli*.** Chemically competent *E. coli* BL21 DE3 RIPL (Agilent Technologies) were transformed with pET28a SnoopTagJr-MBP, pQE80L TriCatcher-ELP, pQE80L TriCatcher-RGDSP, pQE80L TriCatcher-MMP, pQE80L TriCatcher-RGDSP-MMP, pQE-BB, pQE80L SpyTag-ELP-SpyTag or pQ80L SpyTag-ELP-SpyTag DK. The cells were plated on LB agar plates with 50 µg/mL kanamycin (pET28a) or 100 µg/mL ampicillin (pQE80L) and 34 µg/mL chloramphenicol. The plates were incubated at 37 °C for 15-18 h. For all constructs but pQE-BB, individual colonies were grown in 12 mL LB medium with 0.8% (w/v) glucose, 50 µg/mL kanamycin (pET28a) or 100 µg/mL ampicillin (pQE80L) and 34 µg/mL chloramphenicol in a 50 mL reaction tube at 37 °C shaking at 200 rpm (Multitron with 2.5 cm throw, Infors AG) for 12-15 h. Afterwards the culture was diluted at least 100× with fresh LB media with 0.8% (w/v) glucose, 50 µg/mL kanamycin (pET28a) or 100 µg/mL ampicillin (pQE80L) and grown in a 2 L strongly-baffled plastic flasks at 37 °C shaking at 210 rpm. The culture was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (Fluorochem, UK) at $A_{600}$ 0.4-0.6 (pET28a) or $A_{600}$ 0.6-0.8 (pQE80L). Except for pQE80L SpyTag-ELP-SpyTag and pQ80L SpyTag-ELP-SpyTag DK, the temperature was changed to 30 °C after induction. After 4-6 h the cells were harvested and frozen to -80 °C (pET28a) or directly further processed (pQE80L). For pQE-BB, individual colonies from LB agar plates were picked to inoculate 1 L of autoinduction medium (AIML0205 from Formedium) with 100 µg/mL ampicillin in 2 L strongly-baffled plastic flasks and the culture grown at 30 °C with shaking at 200 rpm for 24 h. BB had a yield of 135 mg purified protein per liter of culture. SpyTag-ELP-SpyTag and SpyTag-ELP-SpyTag DK had a typical yield of 30-40 mg purified protein per liter of culture. The TriCatchers had a typical yield of 20-25 mg purified protein per liter of culture.

**Protein expression in mammalian cells.** E-cadherin-SnoopTagJr, EpCAM-SnoopTagJr and EpCAM were expressed in HEK293T cells grown in T175 adhesive culture flasks (Corning) in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with 10% (v/v) Fetal Bovine Serum (Sigma-Aldrich), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL

streptomycin (Thermo Fisher Scientific). HEK 293T cells were seeded into Falcon T875 5-layer flasks (Corning) before transfection. The plasmids were prepared using the ZymoPURE Plasmid Maxiprep Kit (Zymo Research). At 50% confluency, cells were transfected by transferring into serum free medium (DMEM high glucose, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes) with 4 µg/mL plasmid using 7.5 mL per layer in the flask. After 15 min, an additional 2.5 mL serum free medium with 36 µg/mL polyethyleneimine (Sigma-Aldrich) was added. After 16-24 h, another 10 mL of serum free medium with 4.4 µM valproic acid (Sigma-Aldrich) was added and cells were incubated for another 6 d. The supernatant was harvested, EDTA-free Protease Inhibitor Cocktail added according to the manufacturer's recommendation (F. Hoffmann-La Roche AG) and the sample was spun at 220 g for 3 min to remove cell debris. After another filtration through a 0.45 µm syringe filter, 25% Ni-NTA binding buffer was added to adjust the pH for Ni-NTA affinity purification.

**Affinity Purification.** SnoopTagJr-MBP, E-cadherin-SnoopTagJr, EpCAM-SnoopTagJr and EpCAM were purified by standard methods on Ni-NTA (Qiagen) in 50 mM Tris•HCl, 300 mM NaCl, pH 7.8, containing additionally 0.1 g/L $CaCl_2$ and $MgCl_2$ for E-cadherin-SnoopTagJr, EpCAM-SnoopTagJr and EpCAM. Proteins were then dialyzed thrice for at least 3 h each at 4 °C. SnoopTagJr-MBP and SnoopTagJr-mEGFP were dialyzed against PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic dihydrate, 1.8 mM potassium phosphate monobasic, pH adjusted to 7.4 with sodium hydroxide). E-cadherin-SnoopTagJr, EpCAM-SnoopTagJr and EpCAM were dialyzed against PBS containing 0.1 g/L $CaCl_2$ and $MgCl_2$ and concentrated using a Vivaspin 6 centrifugal concentrator with 10 kDa cutoff (GE Healthcare). All proteins were flash frozen in ethanol/dry-ice and stored at −80 °C.

**Inverse Transition Cycling.** TriCatcher-ELP, TriCatcher-RGDSP, TriCatcher-MMP, TriCatcher-RGDSP-MMP, BB (SpyCatcher-ELP-SpyCatcher), SpyTag-ELP-SpyTag and SpyTag-ELP-SpyTag DK were purified using the reversible phase transition property of the ELP.[20] After lysing the cells using sonication, cell debris was removed by centrifugation at 37,500 g for 30 min at 4 °C. For all proteins except BB, unwanted *E. coli* proteins were precipitated by adjusting the pH to 2.2 in 100 mM glycine.[21] Acidified solution was incubated in a rolling tube at 4 °C for 12-15 h. Afterwards, solution was spun again at 37,500 g for 30 min at 4 °C to remove precipitate. After neutralization with sodium hydroxide, the first inverse transition cycling starts by adding 2.5 mM (for SpyTag constructs) or 4.5 mM (for TriCatcher constructs) NaCl to precipitate the target ELP-bearing protein at 25 °C for 15-30 min. Precipitated protein was centrifuged at 25 °C for 15 min at 4,800 g. After the supernatant was discarded, the protein was re-suspended in ice-cold 100 mM Tris•HCl pH 8.0 while rolling at 4 °C until all precipitates were dissolved. Protein solution was centrifuged at 4 °C for 15 min at 4,800 g and supernatant was transferred to new 50 mL tube. Protein concentration was determined from $A_{280}$ using the extinction coefficient calculated by ExPASy ProtParam. The inverse transition cycle was repeated for all constructs. For the TriCatcher constructs, the protein was dissolved at ~10 mg/mL at the end of a third cycle in 100 mM Tris•HCl pH 8.0 with 15 mM octyl β-D-glucopyranoside (Sigma-Aldrich). For BB the acidification was skipped (because BB precipitated at acidic pH) and inverse transition cycling started with 4.5 mM NaCl as described for TriCatcher constructs (using shaking instead of rolling to resuspend the protein pellet). After four cycles the protein solution was centrifuged at 4 °C for 15 min at 4,800 g in 15 mL tubes (instead of 50 mL tubes) to allow better precipitation of insoluble impurities. The supernatant was transferred to a new 50 mL tube and octyl β-D-glucopyranoside added to a final concentration of 15 mM to prevent hydrophobic impurities from binding to BB. The inverse transition cycling of BB was repeated another two times under those modified conditions (15 mL tubes and octyl β-D-glucopyranoside). After six cycles the protein solution was centrifuged at 4 °C for 30 min at 16,900 g in 1.5 mL tubes (instead of 15 mL tubes) to allow even better precipitation of insoluble impurities. After another inverse transition cycle, BB was precipitated using 3.5 M

NaCl (instead of 4.5 M NaCl) to remove impurities carried over at higher salt concentrations and then proceeded as described before to re-solubilize the protein. Overall eight inverse transition cycles were performed for BB with increasing stringency concerning higher centrifugation speed and we used octyl β-D-glucopyranoside to remove impurities. Following inverse transition cycling purification, BB was dialyzed five times against MilliQ for at least 3 h at 4 °C. TriCatcher and SpyTag constructs were dialyzed thrice for at least 3 h each at 4 °C, with TriCatcher constructs dialyzed against PBS. SpyTag constructs were dialyzed against 50 mM ammonium acetate. TriCatcher constructs were flash frozen in ethanol/dry-ice and stored at −80 °C. SpyTag constructs and BB (after sterile filtration through a 0.22 µm syringe filter in a cell culture hood) were flash-frozen in ethanol/dry-ice, lyophilized for at least 2 d using a BenchTop K lyophilizer from VirTis at -60 to -80 °C and 10 to 50 µbar, and stored at −80 °C.

**Synthesis of HA-SpyTag.** The protocol for synthesizing HA-SpyTag is a combination of previous methods.[22-24] Every step was performed at 25 °C unless stated. 400 mg of hyaluronic acid (HA) (242 kDa, sodium salt, Lifecore Biomedical LLC) was dissolved in 40 mL dd$H_2O$. 5 g Dowex 50WX8 resin (200-400 mesh, Acros Organics) was washed five times with 50 mL dd$H_2O$ by centrifugation at 4,800 g for 10 min (Multifuge X3R). 6.4 mL of 40% tetrabutylammonium hydroxide solution (TBA) (Sigma-Aldrich) was added and mixed on a shaker for 30 min. Resin was washed again five times with 50 mL dd$H_2O$ by centrifugation at 4,800 g for 10 min. Dissolved HA solution was added to the resin and incubated on a shaker for 3 h. Solutions were filtered to remove the resin from the HA TBA salt (HA-TBA). HA-TBA solution was flash frozen in ethanol/dry-ice, lyophilized for at least 2 d (as above) and stored at −20 °C.

HA-TBA was dissolved in DMSO at 17 mg/mL. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, AppliChem GmbH) and 1-hydroxybenzotriazole hydrate (HOBt, Sigma-Aldrich) were dissolved in DMSO and added to the HA-TBA solution in equal molar ratio to HA-TBA monomer (638.8 Da) sequentially while vortexing. After 15 min incubating on the shaker, *N*-(2-Aminoethyl)maleimide (as the trifluoroacetate salt, Sigma-Aldrich) was added dissolved in DMSO to the now activated HA and further incubated for 30 min while shaking. The maleimide-functionalized HA (HA-mal) was dialyzed three times against dd$H_2O$ at pH 5.0, three times against dd$H_2O$ at pH 5.0 with 200 mM NaCl to disturb ionic interactions of the activators sticking to HA-mal, and three final times against dd$H_2O$ at pH 5.0 each for 1 h at 4 °C. Following dialysis, HA-mal was stored at −80 °C before coupling to SpyTag-ELP-SpyTag.

To test for maleimide-functionalization of HA, Ellman's reagent was used. First, different concentrations of β-mercaptoethanol (0.3 to 10 mM) were added to HA-mal or PBS.[25] Then Ellman's reagent (Sigma-Aldrich) at 4 mg/mL was used to detect the amount of thiols in all solutions. The signal was measured from $A_{412}$ using the NanoDrop ND-1000. As maleimide reacts with the thiol of β-mercaptoethanol, the solutions containing HA-mal give a lower signal than solutions with just β-mercaptoethanol at the same concentration. Since the concentration of β-mercaptoethanol was known, the difference in signal intensity translates directly to the molar concentration of maleimide on HA. Taking the known concentration of HA used for the maleimide functionalization and the molecular weight of an HA disaccharide unit of 379.32 Da (every HA disaccharide unit has only one carboxylic group), the percentage of functionalized HA disaccharide units could be calculated. The average amount of maleimide on HA determined using this method was 10-20%.

Purified and lyophilized SpyTag-ELP-SpyTag was dissolved in PBS at 10 mg/mL and filtered through a 0.45 µm syringe filter. Ten times the molar amount on tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Thermo Fisher Scientific) in PBS, pH adjusted to 7.4, was added to SpyTag-ELP-SpyTag and incubated at 4 °C for 30 min shaking. To inactivate unreacted TCEP (which can also react with maleimide), ten times the molar amount of TCEP to 4-azidobenzoic acid was added (~0.2 M in tert-butyl methyl ether, Sigma-Aldrich) and the solution was further incubated with shaking at 4 °C for 30 min.[26] Then HA-mal was filtered through a 0.45 µm syringe filter and added to the SpyTag-ELP-

SpyTag solution (in this order) in equal weight ratios and incubated rotating at 4 °C for 2 h, forming HA-SpyTag. To prevent future retro-Michael reaction, the conjugate was dialyzed against 50 mM Tris•HCl pH 9.5 with 4 mM ethanolamine for 15-18 h at 25 °C to open the maleimide ring.[27, 28] Ethanolamine was added to protect lysine side chains on SpyTag-ELP-SpyTag, because maleimide can react with amino groups at basic pH. In the next step, diamide (Sigma-Aldrich) was added to a final concentration of 4 mM to dimerize any unreacted SpyTag-ELP-SpyTag by forming disulfide bonds. Then HA-SpyTag was dialyzed thrice against 50 mM ammonium acetate for at least 3 h each. Before lyophilization for at least 3 d (as above), the HA-SpyTag solution was sterile-filtered through a 0.22 µm syringe filter in the cell culture hood. The powder was stored at -80 °C.

**SDS-PAGE.** SDS-PAGE was performed at 180 V on Tris−glycine gels using an XCell SureLock system (Thermo Fisher Scientific). SDS loading buffer (6×) was 20% (v/v) glycerol, 100 mM Tris·HCl, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue at pH 6.8, and 1% (w/v) dithiothreitol was included for reducing gels. Samples were heated by PCR machine (C1000 ThermalCycler from Bio-Rad) with SDS-loading buffer for 3 min at 95 °C. Gels were stained with InstantBlue Coomassie stain (Expedeon) and imaged using a ChemiDoc XRS imager and QuantityOne (version 4.6) software (Bio-Rad). Note that some gel mobilities deviate from the molecular weight because of the branched nature of isopeptide-linked adducts or because of glycosylation.

**Test for Isopeptide Bond Formation.** TriCatcher constructs at 160 µM were reacted with 320 µM SpyTag-ELP-SpyTag DK at molar ratio of 1:2 in PBS at 4 °C for 12 h. The reaction was stopped with 6× SDS-PAGE loading buffer and heating for 3 min at 95 °C. TriCatcher-RGDSP at 172 µM was incubated with E-cadherin-SnoopTagJr, EpCAM-SnoopTagJr or EpCAM in a molar ratio of 5:1. TriCatcher at 139 µM was incubated with SnoopTagJr-MBP in a molar ratio of 5:1 and 2:1.. The mixture was incubated in PBS at 4 °C for 12 h. Protein stock concentrations were: SnoopTagJr-MBP, 721 µM; E-cadherin-SnoopTagJr, 82.7 µM; EpCAM-SnoopTagJr, 89.9 µM; EpCAM, 97.5 µM. The reaction was stopped with 6× SDS-PAGE loading buffer and heating for 3 min at 95 °C. For the time-course of isopeptide bond formation between SnoopCatcher (10 µM) and SnoopTag-MBP or SnoopTagJr-MBP (5 µM), proteins were mixed at 25 °C in 0.2 mL PCR reaction tubes. After the indicated time, the reaction was quenched by adding 6× SDS-PAGE loading buffer and heating for 3 min at 95 °C. Band intensities were quantified and intensities were determined using a Gel Doc XR imager and Image Lab 6.0 software (Bio-Rad). % Tag reacted was defined as 100×[1-(Tag intensity with SnoopCatcher incubation]/(Tag intensity without SnoopCatcher)]. SnoopTag-MBP and SnoopTagJr-MBP were purified as previously described.[15]

**Assembly of Hydrogel Network and Functionalization.** The concentrations of the proteins involved in hydrogel formation were assessed using the Proteoquant BCA assay (Expedeon) to assure precise molar ratios. HA-SpyTag was dissolved in PBS or DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes at 4 °C, flash-frozen in ethanol/dry-ice and stored at −80 °C. The average concentration of soluble HA-SpyTag was 9 mg/mL in PBS and 7 mg/mL in medium. Stock concentrations of TriCatcher constructs ranged from 10 to 11 mg/mL. TriCatcher-RGDSP coupled to SnoopTagJr-MBP was prepared as described under “Test for Isopeptide Bond Formation”. BB was dissolved in PBS or DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes at 4 °C and aliquoted under sterile conditions, flash frozen in ethanol/dry-ice and stored at −80 °C. The average concentration of soluble BB was 104.5 mg/mL in PBS and 88.7 mg/mL in medium. Hydrogels using only TriCatcher constructs and HA-SpyTag were prepared in two different concentrations, 80 µM and 53.3 µM final concentration of TriCatcher constructs and HA-SpyTag. Additionally, hydrogels were prepared using BB and HA-SpyTag at 80 µM or 120 µM each. Hydrogels made of combinations of 72 µM BB and 48 µM TriCatcher-RGDSP (with and without coupling to SnoopTagJr-MBP) were formed with 120 µM HA-SpyTag. When analyzing the

properties of the hydrogels, sometimes 25% of the overall hydrogel volume was DMEM high glucose with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin to mimic embedding of cells in medium with FBS. The rest of the volume was PBS. Hydrogels were formed in the following order. HA-SpyTag, growth medium or cells, and PBS were mixed first, then TriCatchers or a combination of BB and TriCatcher-RGDSP were added while the open tube was vortexed at 750 rpm for a few seconds (using a STARLAB vortex). BB and TriCatcher-RGDSP (with and without coupling to SnoopTagJr-MBP) were mixed first before adding to HA-SpyTag. TriCatchers were maintained on ice, where they had the highest solubility, before adding to the mixture. After mixing all hydrogel components together, the hydrogel was aliquoted if needed and left to gel for 30 min at 37 °C with 95% humidity and 5% $CO_2$. After gelation, PBS or DMEM high glucose with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin was added and hydrogels further incubated at 37 °C with 95% humidity and 5% $CO_2$.

**Validation that Hydrogel Formation was Isopeptide Bond-Dependent.** SpyTag peptide (GAHIVMVDAYKPTK) was synthesized by Insight Biotechnology, UK. TriCatcher-RGDSP (50 µL) at 172 µM was incubated with twice the molar quantity of SpyTag peptide in DMSO (2.5 µL, 6.54 mM) for 1 h at 4 °C. As a control, TriCatcher-RGDSP at the same concentration was incubated with 2.5 µL pure DMSO. Immediately afterwards, hydrogel at high concentration was formed in a 1.5 mL reaction tube with HA-SpyTag in PBS. Hydrogel was incubated for 30 min at 37 °C. Following this incubation step, 1 mL of PBS was added to both tubes (with and without pre-incubation with SpyTag peptide), tubes inverted and a picture of the hydrogel was taken immediately using a Microsoft Lumia 950 mobile phone.

**Rheological Characterization of the Hydrogels.** Rheological measurements were performed using the strain-controlled rheometer Paar Physica MCR 301 and a geometry adaptor with 25 mm diameter (both Anton-Paar). The hydrogel was prepared with HA-SpyTag and BB in DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes. For hydrogels consisting of TriCatcher constructs and HA-SpyTag the final volume contained 25% DMEM high glucose with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin to mimic embedding cells into hydrogel at a later stage. This growth medium was replaced once with PBS to check the impact of the growth medium. For the sample with 80 µM TriCatcher-RGDSP alone, HA-SpyTag was replaced with DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes. Additionally, BB or HA-SpyTag individually at 120 µM were measured as a control. Measurements were performed at 23 °C. All hydrogels were incubated using an almost closed environment containing a water reservoir around the geometry adaptor to avoid evaporation. The evolution of storage (G') and loss modulus (G'') directly after mixing of the hydrogel components was measured using 1 Hz frequency and 1% deformation for 30 min. A subsequent frequency sweep was acquired using 1% deformation and a range from 0.1 to 10 Hz. Following the frequency sweep, strain sweeps were done with 1 Hz frequency over a range of 0.1 to 1000%.

**Analysis of Hydrogel Swelling.** Freshly formed hydrogels (50 µL) in 0.5 mL reaction tubes were incubated for 30 min at 37 °C, 95% humidity and 5% $CO_2$ to mimic cell culture conditions. Reaction tubes were weighed on a micro-balance (College-B B154, Mettler Toledo) before and after hydrogel formation. Afterwards 0.5 mL of either PBS (if hydrogel was formed in pure PBS) or DMEM high glucose with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (if hydrogel contained already medium) was added and hydrogels were further incubated at 37 °C, 95% humidity and 5% $CO_2$ for 24 h. Hydrogels containing medium were prepared using HA-SpyTag in DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes and 25% DMEM high glucose with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Afterwards, supernatant was removed with a paper tissue and the reaction tube was weighed to calculate the value for the swollen hydrogel by subtracting the

weight of the empty tube documented beforehand.

**Analysis of Hydrogel Erosion.** Freshly formed hydrogels (50 µL in pure PBS) in 0.5 mL reaction tubes were incubated for 30 min at 37 °C, 95% humidity and 5% $CO_2$ to mimic cell culture conditions. Afterwards 0.5 mL of PBS was added and hydrogels further incubated at 37 °C, 95% humidity and 5% $CO_2$. 10 µL aliquots were taken after 1 d and then every three days. The amount of protein released into PBS was determined immediately after taking the sample using a Proteoquant BCA assay (Expedeon) and a SpectraMAX M3 plate-reader (Molecular Devices).

**Embedding Mouse 3T3 Fibroblasts in Hydrogels.** The mouse 3T3 fibroblast cell-line was grown at 37 °C with 95% humidity and 5% $CO_2$ in DMEM high glucose with 2 mM L-glutamine (Thermo Fisher Scientific), 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) in T175 adhesive culture flasks (Corning). At ~75% confluence, cells were detached with 1 mL trypsin-EDTA (0.05%) solution (Thermo Fisher Scientific), followed by addition of 9 mL media to neutralize the trypsin. The cell suspension was transferred to a 15 mL centrifuge tube and spun at 100 g for 3 min. After removal of the medium, pelleted cells were re-suspended in 10 mL full medium and counted using a hemocytometer. Hydrogel was formed with HA-SpyTag and BB in DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 25 mM Hepes. TriCatcher constructs were sterile-filtered (Costar® Spin-X® centrifuge tube filter – 0.22 µm cellulose acetate sterile and RNase/DNase free, Corning). PBS and HA-SpyTag was mixed first (until mixing, HA-SpyTag was kept on ice), then 6.2% of final volume in cells (in medium with FBS) was added ($10^6$ cells/mL final). Last, TriCatcher constructs or a mixture of BB and TriCatcher-RGDSP were added during vortexing (the TriCatcher construct and BB was kept on ice until this point). BB and TriCatcher-RGDSP (with and without coupling to SnoopTagJr-MBP) were mixed first before adding to HA-SpyTag. 120 µM HA-SpyTag was used for all hydrogels. Either 120 µM BB was used or 72 µM BB with 48 µM TriCatcher-RGDSP (with and without coupling of TriCatcher-RGDSP to SnoopTagJr-MBP). SnoopTagJr-MBP was coupled to TriCatcher-RGDSP at a ratio of 1:2 before hydrogel formation, leading to a final concentration of 24 µM SnoopTagJr-MBP in the hydrogel.

Mouse 3T3 fibroblasts at $10^6$ cell/mL were embedded in hydrogels of the following compositions: 80 µM HA-SpyTag and 80 µM TriCatcher (for ELP, RGDSP, MMP, RGDSP-MMP); 120 µM HA-SpyTag and 120 µM BB or 72 µM BB with 48 µM TriCather-RGDSP or 72 µM BB with 48 µM TriCatcher-RGDSP and 24 µM SnoopTagJr-MBP. SnoopTagJr-MBP was coupled to TriCatcher-RGDSP at a ratio of 1:2 before hydrogel formation. After culture for 1 d, samples were analyzed by confocal microscopy. Immediately afterwards, 10 µL of hydrogel was pipetted into µ-angiogenesis slides (ibidi GmbH) in triplicate. After allowing hydrogel formation for 30 min at 25 °C, 50 µL of medium with FBS was added to each well and the slide was put back in the cell-culture incubator for 24 h before assay.

**Hanging Drop Culture of MCF 10A Mammary Epithelial Cells in Hydrogels.** The non-malignant MCF 10A human mammary epithelial cell-line (cat. No. CRL10317 from ATCC) was grown at 37 °C with 95% humidity and 5% $CO_2$ in DMEM/F12 with 5% (v/v) horse serum, 2 mM L-glutamine (Thermo Fisher Scientific), 20 ng/mL epidermal growth factor (PeproTech), 0.5 µg/mL hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin from *Vibrio cholerae* (Sigma-Aldrich), 10 µg/mL insulin expressed in *Saccharomyces cerevisiae* (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) (MCF 10A growth medium) in T175 adhesive culture flasks (Corning).[29] At ~75% confluence, cells were detached with 1 mL of a trypsin-EDTA (0.05%) solution (Thermo Fisher Scientific), followed by addition of 9 mL media to neutralize the trypsin. The cell suspension was transferred to a 15 mL centrifuge tube and spun at 100 g for 3 min. After removal of the medium, pelleted cells were re-suspended in 10 mL full medium and counted using a hemocytometer. Cells were diluted to 20,000 cells/mL in full medium and 330 drops of 5 µL were pipetted on the lid of a 10 cm × 10 cm petri dish and inverted. One petri dish per sample was prepared. With 12

mL sterile PBS in the bottom part of the petri dish, the cells were placed in the cell-culture incubator overnight for sphere formation.

**Embedding MCF 10A Mammary Epithelial Cell Spheres in Hydrogel.** 6 mL MCF 10A growth medium was used to wash cell spheres off the lid of the petri dish after overnight incubation of the hanging drop culture. The suspension with the spheres was sucked into a 5 mL serological pipette and the tip of the serological pipette closed by inserting tightly into a 0.5 mL microcentrifuge tube. After occasionally rocking the serological pipette held vertical for 15 min, the spheres settled in to the bottom of the microcentrifuge tube. The serological pipette was removed and the liquid in the reaction tube adjusted to the volume needed for embedding the spheres in the hydrogel. Hydrogel was formed with HA-SpyTag in PBS pH 7.4. TriCatchers coupled to SnoopTagJr-ligands were prepared as described under “Test for Isopeptide Bond Formation”. TriCatcher constructs (with and without coupled SnoopTagJr-ligands) were sterile-filtered (Costar® Spin-X® centrifuge tube filter – 0.22 µm cellulose acetate sterile and RNase/DNase free, Corning). PBS and HA-SpyTag were mixed to the suspension of spheres first (until mixing, HA-SpyTag was kept on ice) and spheres were resuspended by pipetting up and down. Then TriCatcher constructs were added during vortexing (TriCatcher construct was kept on ice until this point).

Hydrogels were prepared with 53.3 µM TriCatcher and HA-SpyTag and 10.7 µM ligand. Two examples for EpCAM-SnoopTagJr and EpCAM in solution are shown (one containing a sphere and one with no sphere remaining). Immediately afterwards, 10 µL of hydrogel was pipetted into µ-angiogenesis slides (ibidi GmbH). After letting the hydrogels form for 30 min at 25 °C, 50 µL of MCF 10A growth medium was added to each well and the slide was put back in the incubator. For four days, 10 µL of fresh MCF 10A growth medium was added per well per day. After 6 d, the spheres were imaged.

**Fluorescence microscopy.** For mouse 3T3 fibroblasts, medium was removed and the hydrogel was washed twice with PBS containing 0.1 mg/mL calcium chloride (50 µL). Live/dead reagent in PBS (with 0.1 g/L $CaCl_2$ and $MgCl_2$), contained 6 µM calcein AM ester (Sigma-Aldrich) and 6 µM propidium iodide (Sigma-Aldrich), was added to cells, removed and added again, followed by incubating for 30 min at 37 °C with 95% humidity and 5% $CO_2$. Afterwards, hydrogel was washed with PBS and twice with DMEM high glucose with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 mM Hepes and imaged in the same medium. A 100 µm thickness z-stack was taken with 1 µm distance between the slices. Using ImageJ 1.52c the threshold distinguishing cells from the background was calculated from the whole stack using the “MaxEntropy” setting in the threshold option.[30] The “Li” setting was also used for calcein AM ester staining. The percentage of live cells was measured using the Fiji macro 3D object counter 2.0.1 based on the total number of cells compared to cells stained with propidium iodide.{S., 2006 #12820}

For MCF 10A mammary epithelial cell spheres, 20 µM calcein AM ester and 2 µM propidium iodide were added to medium in a final concentration. Spheres were imaged after 30 min incubation at 25 °C. Using ImageJ 1.52c the threshold distinguishing cells from the background was calculated from the whole stack using the “Triangle” setting in the threshold option.[30]

Cells were imaged with a spinning-disc confocal microscope (Ultra-VIEW VoX, PerkinElmer), mounted on an IX81 microscope with 10×/0.4 air UPlanSApo objective (Olympus Corporation, Tokyo, Japan), and an electron-multiplying charge-coupled device camera (ImagEM, Hamamatsu Photonics).

To stain for nuclei, cells were fixed with 4% (w/v) formaldehyde in PBS containing 0.1 mg/mL calcium chloride and 0.1 mg/mL magnesium chloride (50 µL) for 30 min, washed twice with PBS containing 3% (w/v) Bovine Serum Albumin (50 µL) and once with PBS (50 µL). Nuclei were stained with 10 µM Hoechst 33342 (50 µL) (Thermo Fisher Scientific) for 1 h. Finally, cells were washed three times with PBS (50 µL), incubating the sample in-between washes for 45 min. The staining was visualized with a spinning-disc confocal

microscope as above. A 100 µm thickness z-stack was taken with 1 µm distance between the slices.

MCF 10A spheres were detected based on Hoechst 33342 staining after 6 d of culture in the hydrogel. To measure the size of MCF 10A spheres, the ROI tool in OMERO.insight version 5.3.5 was used to measure the longest and shortest axis of the sphere.[31] The mean of those two axes was plotted and used for the statistical analysis.

**Hydrogel movie.** The hydrogel was formed with 80 µM of HA-SpyTag and TriCatcher-RGDSP as described in in the method section "Assembly of Hydrogel Network and Functionalization". Toluidine blue dye was used to facilitate visibility of HA-SpyTag and the formed hydrogel. The vortex was set to 5,000 rpm. Directly after hydrogel formation the tube was inverted and flicked. Immediately afterwards, PBS with resazurin was added to the hydrogel and the tube was vortexed again.

**Statistical analysis.** GraphPad Prism 7.04 was used for statistical analysis. The analysis used was one-way ANOVA comparing the mean between data sets, corrected by controlling the false discovery rate using the two-stage step-up method of Benjamini, Krieger and Yekutieli. The following number of replicates were used for the detection of spheres after 6 days of culture in the hydrogels (Figure 4b): control (n=7), EpCAM-SnoopTagJr (n=4), EpCAM in solution (n=4) and SnoopTagJr-MBP (n=3). The size of the spheres after 6 d of culture in the hydrogels (Figure 4c) was taken from the same datasets, measuring the size of all spheres detected: control (n=306), EpCAM-SnoopTagJr (n=41), EpCAM in solution (n=161) and SnoopTagJr-MBP (n=187).

## Results and Discussion

As a central building block, the protein TriCatcher was designed, comprising two SpyCatcher moieties linked by an ELP and a C-terminal SnoopCatcher (Figure 1b and S1). We generated three variants beyond TriCatcher-ELP (Figure 1c). TriCatcher-RGDSP has the RGDSP motif in the center to promote cell adhesion through integrins.[32] TriCatcher-MMP has a cleavage site for matrix metalloproteinases (MMPs), facilitating hydrogel turn-over as cells grow.[17] TriCatcher-RGDSP-MMP bears both motifs (Figure 1c). For initial validation of the reactivity of SpyCatcher moieties, each TriCatcher variant was incubated with SpyTag-ELP-SpyTag DK (with the second SpyTag inactive from Asp to Lys mutation) (Figure S2 and S3). We observed quantitative coupling of each TriCatcher to two copies of SpyTag-ELP-SpyTag DK (Figure S3). Therefore, TriCatchers can be used as cross-linkers with SpyTag ligands. All protein components of the network (TriCatchers and SpyTag-ELP-SpyTag) were expressed in *Escherichia coli* and purified by inverse transition cycling: the reversible precipitation of ELPs either when heated above their lower critical solution temperature or by addition of NaCl.[20] Inverse transition cycling is easily scalable compared to affinity resin-purification.

Hydrogels formed previously using SpyTag/SpyCatcher had solid content of 5-15%.[11-13] Since a low hydrogel storage modulus is required for many cell-types,[33] we reduced solid content 10-fold using the polysaccharide hyaluronan (HA, 242 kDa molecular weight) as an additional hydrogel component. HA is an abundant part of the extracellular matrix, modulating cell behavior and migration.[34] Linking SpyTag-ELP-SpyTag to HA would also increase the valency, to overcome the low cross-linking efficiency of ELP-only SpyTag/SpyCatcher hydrogels resulting from protein loops forming instead of networks.[11] Coupling of SpyTag-ELP-SpyTag (with a central cysteine) to HA-maleimide via Michael-type addition was validated by SDS-PAGE (Figure S4 and S5a).

We tested hydrogel formation by mixing TriCatcher and HA-SpyTag both at 53.3 µM (low concentration, solid content 0.55%) or 80 µM (high concentration, solid content 0.84%). Testing in PBS, we found that gelation occurred in a few seconds (Movie S1). However, with this rapid rate, aliquoting a cell mix would not be possible. In cell medium (± fetal bovine serum), hydrogel formation was delayed by 3-5 min, which allowed further aliquoting of

samples (vide infra) and should also minimize disturbance to cell behavior. When TriCatchers were pre-incubated with free SpyTag, peptide hydrogel formation was abolished, validating that gelling depended on isopeptide bond formation (Figure 2a).

Having established network formation, we next explored functionalization of the hydrogel. SnoopTagJr is a mutant of SnoopTag we recently generated for SnoopLigase peptide-peptide ligation.[16] A time-course of the reaction of SnoopTag-MBP or SnoopTagJr-MBP revealed that SnoopTagJr also led to faster conjugate formation with SnoopCatcher (Figure S5b). Therefore, SnoopTagJr was used in all our subsequent studies. After 12 h at 4 °C, we showed quantitative coupling of the SnoopTagJr-MBP to TriCatcher (Figure 2b). We then validated coupling to two complex mammalian adhesion proteins, important for cancer cell development. Epithelial cell adhesion molecule (EpCAM) is a common marker on cancer cells but is also present on healthy mammary epithelial cells.[35, 36] EpCAM activation may increase cell migration, inducing epithelial-mesenchymal transition and suppressing cell-cell adhesion by E-cadherin.[35, 37-40] We linked SnoopTagJr to the C-terminus of the extracellular region of EpCAM or E-cadherin and purified these proteins from HEK293T cells. EpCAM contains heterogeneous glycosylation, leading to the expected diffuse banding on SDS-PAGE (Figure S6).[37] Both proteins showed efficient reaction with TriCatcher (Figure S6). These results indicate that SnoopTagJr/SnoopCatcher could be suited for modular functionalization of hydrogels, with the goal of mimicking cell-cell interactions between epithelial and cancer cells.

To mimic the cell-cell interface, the hydrogel's mechanical behavior must be optimized. Mammary epithelial cells grow in hydrogels of low storage modulus (~30 Pa), otherwise the cells show a cancerous phenotype.[33] Hydrogels were analyzed by dynamic shear rheology in growth medium to mimic culture conditions. As shown in Figure 2c, S7a and S8a, hydrogel formation occurred in 3-5 min (2 min were needed for applying the hydrogel to the instrument). Since the SpyTag/SpyCatcher reaction driving the covalent cross-linking is fast, slight variations in the initial mixing can lead to noticeable differences in initial gelation, comparing high and low hydrogel concentrations (Figure 2c). There was little change in modulus after 30 min, so subsequent experiments were performed after 30 min of gelation (Figure 2c, S7a and S8a). Hydrogel storage modulus ranged from ~10 Pa for low concentration to 30 Pa for high concentration of TriCatcher/HA-SpyTag, well suited for embedding mammary epithelial cells.[33] Interestingly, strain sweep experiments revealed that the hydrogel had a wide elastic regime (Figure 2d, S7b and S8b). G' (storage modulus) and G'' (loss modulus) were constant between 0.1 and 3 Hz (Figure 2e, S7c and S8c), supporting the hydrogel's elastic nature.[41] Functionalization with SnoopTagJr-MBP did not lead to substantially different modulus (Figure S8). We further explored how to increase the storage modulus of the hydrogel, while retaining the ability to modify the hydrogel with SnoopTagJr-protein ligands. Since the mechanical properties are limited by the solubility of the TriCatcher and HA-SpyTag constructs, more soluble hydrogel components are needed to reach higher storage moduli. BB (SpyCatcher-ELP-SpyCatcher) has a solubility of 100 mg/mL.[11] We purified BB using inverse transition cycling (Figure S9a). Neither HA-SpyTag nor TriCatcher nor BB alone led to hydrogel formation (Figure S9b). A storage modulus of 51 Pa was reached from reacting 120 µM BB with HA-SpyTag (Figure S10). By replacing part of the BB fraction with TriCatcher, the ability to functionalize the hydrogel with SnoopTagJr-protein ligands was restored, keeping the higher storage modulus (Figure S11).

Hydrogel erosion was analyzed from the appearance of proteins in the supernatant at 37 °C.[11] Hydrogels prepared at lower concentration eroded faster than at higher concentration (Figure 3a). RGDSP or MMP-cleavage sites had only a minor effect on hydrogel stability over time (Figure 3a). Functionalization with SnoopTagJr-MBP did not influence hydrogel erosion (Figure 3a). Over 1 week there was ~30% hydrogel erosion, sufficient resilience for our analysis of mammary cell behavior. Hydrogel swelling changes the concentration of ligands coupled to the hydrogel and can place additional force on the cells. For hydrogels formed at different concentrations or in different media, only a low level of swelling was found (19-31%) (Figure 3b and 3c).

We embedded mouse 3T3 fibroblasts in low-concentration hydrogels containing each TriCatcher to evaluate biocompatibility. There was no spreading of the fibroblasts without RGDSP after 1 day of culture (Figure 4). With RGDSP, cells could spread within the hydrogel. Cell spreading was not influenced by the MMP-cleavable linker. Staining with calcein AM ester and propidium iodide revealed 93-97% cell viability (Figure S12a). Integrating SnoopTagJr-MBP into the hydrogels containing RGDSP did not change cell spreading (Figure 4). Also, the use of BB and combinations of BB with TriCatcher-RGDSP (with and without coupling to SnoopTagJr-MBP) led to cell spreading and high cell viability (Figure S12b). Overall, ligands could be presented to cells without causing cell spreading in 3D (needed for mammary epithelial cells) and ligands could be integrated in the hydrogel without compromising integrin-mediated cell adhesion.

Solid tumors depend on a range of mechanical, metabolic and biochemical inputs from neighboring cells.[42, 43] Model systems are crucial to dissect the individual inputs to cancer cell behavior. Non-malignant MCF 10A mammary epithelial spheres are an established model for mammary gland development.[33, 44] The MCF 10A cell-line is normally grown in 2D but, when cultured in hanging drops overnight, the cells spontaneously assembly into spheres (Figure S13a).[45] We hypothesized that we could mimic the cancer cell:healthy cell interface by hydrogel-mediated display of EpCAM. MCF 10A spheres were embedded in a hydrogel of TriCatcher-ELP and HA-SpyTag either with no ligand, EpCAM-SnoopTagJr, soluble EpCAM, or SnoopTagJr-MBP. After 6 days in the hydrogel, cells were analyzed by fluorescence microscopy. All cell clusters were imaged, with spheres defined as multiple cells having a visible spherical boundary around the cell cluster compared to the boundary between individual cells. In hydrogel without ligand, nearly all spheres remained intact, whereas with EpCAM-SnoopTagJr, 77% of spheres dissociated (Figure 5a and 5b). Using soluble EpCAM, more spheres remained, most likely because of EpCAM diffusing into the supernatant. In hydrogels coupled to SnoopTagJr-MBP, the spheres behaved the same as in the ligand-free hydrogel, showing that coupling protein ligands to TriCatcher by itself did not influence sphere development (Figure 5). EpCAM-SnoopTagJr caused the most decrease in MCF 10A sphere size ($p<0.0001$ by one-way ANOVA), followed by soluble EpCAM ($p<0.0001$ by one-way ANOVA). SnoopTagJr-MBP-functionalized hydrogels did not have a significant influence on sphere size compared to the no-ligand control ($p=0.1$ by one-way ANOVA) (Figure 5c). Live/dead staining using calcein AM ester and propidium iodide resulted in only a few percent of propidium iodide-positive cells, showing that the dissociation of cells was not from toxicity of EpCAM (Figure S13b-c).

In summary, we report a hydrogel system based on orthogonal amidation and transamidation by peptides, so that independent reactions can be used for network assembly and network functionalization. Combining an ELP network with a hyaluronan network allows rapid, stable and biocompatible gel formation, triggered by SpyTag/SpyCatcher amide bond formation. The hydrogel demonstrates low swelling, tunable degradation and low storage modulus. Such storage modulus may facilitate ligand-induced clustering on the surface of contacting cells.[33] HA-ELP hydrogels cross-linked by hydrazone bonds have a similar storage modulus at 1% solid content as hydrogels formed by TriCatchers and HA-SpyTag, indicating optimal cross-linking.[46, 47] SnoopTagJr/SnoopCatcher reaction allowed efficient and site-specific coupling of complex protein ligands without interfering with hydrogel mechanical behavior. Fibroblasts could be cultured in the hydrogel with good viability and their spreading was RGDSP-dependent. Anchoring of EpCAM to the hydrogel induced dissociation and spreading of mammary epithelial cells. The modular and efficient functionalization of this hydrogel should provide a powerful platform to screen and dissect the role of cell-surface interactions in controlling cell behavior, relevant to development, disease and organoid engineering.[48, 49]

## Conclusion

We present a hydrogel system, which can be efficiently and specifically functionalized with proteins. Independent hydrogel cross-linking and functionalization was possible by using an orthogonal peptide/protein pair, forming spontaneous amide bonds simply upon mixing. Cell

spreading could be tuned by integrating the RGDSP integrin binding motif into the hydrogel network. The presented hydrogel system allowed us to couple the extracellular domain of EpCAM to the hydrogel to modulate the behavior of human mammary epithelial spheres in 3D culture. Our study provides a reference for how bioorthogonal protein chemistries can advance biomaterial assembly and efficiently explore how individual proteins impact complex cell behavior.

## Acknowledgements

Funding for R.W. was provided by the German Research Foundation (grant number: WI 4668/1-1). We thank Andrew Jefferson, David Pinto, Carina Monico and Maryam Qurashi from the Micron Oxford Advanced Bioimaging Unit (Wellcome Trust Strategic Award, grant number:107457/Z/15/Z) for microscopy support. We also thank Kalin Dragnevski and Marzena Tkaczyk of the Oxford University Laboratory for In-situ Microscopy & Analysis (LIMA) for support with rheology and Irsyad Khairil Anuar (Howarth laboratory) for help with SnoopTagJr.

## Author contribution

R.W. carried out the experiments and analyzed the data. R.W. and M.H. conceived and designed the experiments, interpreted the data and wrote the manuscript.

## Conflicts of interest

M.H. is an author on patent applications covering SpyTag/SpyCatcher (UK Intellectual Property Office EP2534484) and SnoopTag/SnoopCatcher (UK Intellectual Property Office 1509782.7).

## Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

## References

[1] M.W. Tibbitt, R. Langer, Living Biomaterials, Acc Chem Res 50(3) (2017) 508-513.
[2] X. Yu, X. Tang, S.V. Gohil, C.T. Laurencin, Biomaterials for Bone Regenerative Engineering, Adv Healthc Mater 4(9) (2015) 1268-85.
[3] M. Sheikholeslam, M.E.E. Wright, M.G. Jeschke, S. Amini-Nik, Biomaterials for Skin Substitutes, Adv Healthc Mater 7(5) (2018) 1700897.
[4] M.W. Tibbitt, C.B. Rodell, J.A. Burdick, K.S. Anseth, Progress in material design for biomedical applications, Proc Natl Acad Sci U S A 112(47) (2015) 14444-51.
[5] L.J. Vega, M.K. Lee, E.C. Qin, M. Rich, K.Y. Lee, D.H. Kim, H.J. Chung, D.E. Leckband, H. Kong, Three Dimensional Conjugation of Recombinant N-Cadherin to a Hydrogel for In Vitro Anisotropic Neural Growth, J Mater Chem B 4(42) (2016) 6803-6811.
[6] A. Banerjee, M. Howarth, Nanoteamwork: covalent protein assembly beyond duets towards protein ensembles and orchestras, Curr Opin Biotechnol 51 (2017) 16-23.
[7] S.A. Fisher, A.E.G. Baker, M.S. Shoichet, Designing Peptide and Protein Modified Hydrogels: Selecting the Optimal Conjugation Strategy, J Am Chem Soc 139(22) (2017) 7416-7427.
[8] C.M. Madl, L.M. Katz, S.C. Heilshorn, Bio-Orthogonally Crosslinked, Engineered Protein Hydrogels with Tunable Mechanics and Biochemistry for Cell Encapsulation, Adv Funct Mater 26(21) (2016) 3612-3620.
[9] B. Zakeri, J.O. Fierer, E. Celik, E.C. Chittock, U. Schwarz-Linek, V.T. Moy, M. Howarth, Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin, Proc Natl Acad Sci U S A 109(12) (2012) E690-7.
[10] A.H. Keeble, A. Banerjee, M.P. Ferla, S.C. Reddington, I. Anuar, M. Howarth, Evolving Accelerated Amidation by SpyTag/SpyCatcher to Analyze Membrane Dynamics, Angew Chem Int Ed Engl 56(52) (2017) 16521-16525.

[11] F. Sun, W.B. Zhang, A. Mahdavi, F.H. Arnold, D.A. Tirrell, Synthesis of bioactive protein hydrogels by genetically encoded SpyTag-SpyCatcher chemistry, Proc Natl Acad Sci U S A 111(31) (2014) 11269-74.
[12] R. Wang, Z. Yang, J. Luo, I.M. Hsing, F. Sun, B12-dependent photoresponsive protein hydrogels for controlled stem cell/protein release, Proc Natl Acad Sci U S A 114(23) (2017) 5912-5917.
[13] X. Gao, J. Fang, B. Xue, L. Fu, H. Li, Engineering Protein Hydrogels Using SpyCatcher-SpyTag Chemistry, Biomacromolecules 17(9) (2016) 2812-9.
[14] X. Gao, S. Lyu, H. Li, Decorating a Blank Slate Protein Hydrogel: A General and Robust Approach for Functionalizing Protein Hydrogels, Biomacromolecules 18(11) (2017) 3726-3732.
[15] G. Veggiani, T. Nakamura, M.D. Brenner, R.V. Gayet, J. Yan, C.V. Robinson, M. Howarth, Programmable polyproteams built using twin peptide superglues, Proc Natl Acad Sci U S A 113(5) (2016) 1202-7.
[16] C.M. Buldun, J.X. Jean, M.R. Bedford, M. Howarth, SnoopLigase Catalyzes Peptide-Peptide Locking and Enables Solid-Phase Conjugate Isolation, J Am Chem Soc 140(8) (2018) 3008-3018.
[17] J. Patterson, J.A. Hubbell, Enhanced proteolytic degradation of molecularly engineered PEG hydrogels in response to MMP-1 and MMP-2, Biomaterials 31(30) (2010) 7836-45.
[18] K.D. Brune, D.B. Leneghan, I.J. Brian, A.S. Ishizuka, M.F. Bachmann, S.J. Draper, S. Biswas, M. Howarth, Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization, Scientific reports 6 (2016) 19234.
[19] K.C. Miranda, T. Khromykh, P. Christy, T.L. Le, C.J. Gottardi, A.S. Yap, J.L. Stow, R.D. Teasdale, A dileucine motif targets E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells, J Biol Chem 276(25) (2001) 22565-72.
[20] W. Hassouneh, T. Christensen, A. Chilkoti, Elastin-like polypeptides as a purification tag for recombinant proteins, Curr Protoc Protein Sci Chapter 6 (2010) Unit 6 11.
[21] Y.Y. Peng, V. Stoichevska, S. Madsen, L. Howell, G.J. Dumsday, J.A. Werkmeister, J.A. Ramshaw, A simple cost-effective methodology for large-scale purification of recombinant non-animal collagens, Applied microbiology and biotechnology 98(4) (2014) 1807-15.
[22] T.A. Holstlaw, M. Mahomed, L.W. Brier, D.M. Young, N.J. Boudreau, W.M. Jackson, Biopolymer Molecular Weight Can Modulate the Wound Healing Efficacy of Multivalent Sonic Hedgehog-Hyaluronic Acid Conjugates, Biomacromolecules 18(8) (2017) 2350-2359.
[23] Y. Zhang, P. Heher, J. Hilborn, H. Redl, D.A. Ossipov, Hyaluronic acid-fibrin interpenetrating double network hydrogel prepared in situ by orthogonal disulfide cross-linking reaction for biomedical applications, Acta Biomater 38 (2016) 23-32.
[24] J.A. Yang, H. Kim, K. Park, S.K. Hahn, Molecular design of hyaluronic acid hydrogel networks for long-term controlled delivery of human growth hormone, Soft Matter 7(3) (2011) 868-870.
[25] C.K. Riener, G. Kada, H.J. Gruber, Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine, Analytical and bioanalytical chemistry 373(4-5) (2002) 266-76.
[26] M. Henkel, N. Rockendorf, A. Frey, Selective and Efficient Cysteine Conjugation by Maleimides in the Presence of Phosphine Reductants, Bioconjug Chem 27(10) (2016) 2260-2265.
[27] L.N. Tumey, M. Charati, T. He, E. Sousa, D. Ma, X. Han, T. Clark, J. Casavant, F. Loganzo, F. Barletta, J. Lucas, E.I. Graziani, Mild method for succinimide hydrolysis on ADCs: impact on ADC potency, stability, exposure, and efficacy, Bioconjug Chem 25(10) (2014) 1871-80.
[28] D. Shinmi, E. Taguchi, J. Iwano, T. Yamaguchi, K. Masuda, J. Enokizono, Y. Shiraishi, One-Step Conjugation Method for Site-Specific Antibody-Drug Conjugates through Reactive Cysteine-Engineered Antibodies, Bioconjug Chem 27(5) (2016) 1324-31.
[29] J. Debnath, S.K. Muthuswamy, J.S. Brugge, Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures, Methods 30(3) (2003) 256-68.
[30] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A.

Cardona, Fiji: an open-source platform for biological-image analysis, Nat Methods 9(7) (2012) 676-82.
[31] C. Allan, J.M. Burel, J. Moore, C. Blackburn, M. Linkert, S. Loynton, D. Macdonald, W.J. Moore, C. Neves, A. Patterson, M. Porter, A. Tarkowska, B. Loranger, J. Avondo, I. Lagerstedt, L. Lianas, S. Leo, K. Hands, R.T. Hay, A. Patwardhan, C. Best, G.J. Kleywegt, G. Zanetti, J.R. Swedlow, OMERO: flexible, model-driven data management for experimental biology, Nat Methods 9(3) (2012) 245-53.
[32] J.A. Hubbell, Biomaterials in tissue engineering, Biotechnology (N Y) 13(6) (1995) 565-76.
[33] O. Chaudhuri, S.T. Koshy, C. Branco da Cunha, J.W. Shin, C.S. Verbeke, K.H. Allison, D.J. Mooney, Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium, Nat Mater 13(10) (2014) 970-8.
[34] H. Kim, H. Jeong, S. Han, S. Beack, B.W. Hwang, M. Shin, S.S. Oh, S.K. Hahn, Hyaluronate and its derivatives for customized biomedical applications, Biomaterials 123 (2017) 155-171.
[35] M.J. Winter, B. Nagelkerken, A.E. Mertens, H.A. Rees-Bakker, I.H. Briaire-de Bruijn, S.V. Litvinov, Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak, Exp Cell Res 285(1) (2003) 50-8.
[36] D. Maetzel, S. Denzel, B. Mack, M. Canis, P. Went, M. Benk, C. Kieu, P. Papior, P.A. Baeuerle, M. Munz, O. Gires, Nuclear signalling by tumour-associated antigen EpCAM, Nat Cell Biol 11(2) (2009) 162-71.
[37] S.V. Litvinov, M. Balzar, M.J. Winter, H.A.M. Bakker, I.H.B.-d. Bruijn, F. Prins, G.J. Fleuren, S.O. Warnaar, Epithelial Cell Adhesion Molecule (Ep-CAM) Modulates Cell–Cell Interactions Mediated by Classic Cadherins, The Journal of Cell Biology 139(5) (1997) 1337-1348.
[38] M.J. Winter, V. Cirulli, I.H. Briaire-de Bruijn, S.V. Litvinov, Cadherins are regulated by Ep-CAM via phosphaditylinositol-3 kinase, Mol Cell Biochem 302(1-2) (2007) 19-26.
[39] N.V. Sankpal, T.P. Fleming, P.K. Sharma, H.J. Wiedner, W.E. Gillanders, A double-negative feedback loop between EpCAM and ERK contributes to the regulation of epithelial-mesenchymal transition in cancer, Oncogene 36(26) (2017) 3706-3717.
[40] A. Kourtidis, R. Lu, L.J. Pence, P.Z. Anastasiadis, A central role for cadherin signaling in cancer, Exp Cell Res 358(1) (2017) 78-85.
[41] J.M. Zuidema, C.J. Rivet, R.J. Gilbert, F.A. Morrison, A protocol for rheological characterization of hydrogels for tissue engineering strategies, J Biomed Mater Res B Appl Biomater 102(5) (2014) 1063-73.
[42] L. Gu, D.J. Mooney, Biomaterials and emerging anticancer therapeutics: engineering the microenvironment, Nat Rev Cancer 16(1) (2016) 56-66.
[43] M. De Palma, D. Biziato, T.V. Petrova, Microenvironmental regulation of tumour angiogenesis, Nat Rev Cancer 17(8) (2017) 457-474.
[44] M. Nowak, U. Freudenberg, M.V. Tsurkan, C. Werner, K.R. Levental, Modular GAG-matrices to promote mammary epithelial morphogenesis in vitro, Biomaterials 112 (2017) 20-30.
[45] C. Snyman, E. Elliott, An optimized protocol for handling and processing fragile acini cultured with the hanging drop technique, Anal Biochem 419(2) (2011) 348-50.
[46] D. Zhu, H. Wang, P. Trinh, S.C. Heilshorn, F. Yang, Elastin-like protein-hyaluronic acid (ELP-HA) hydrogels with decoupled mechanical and biochemical cues for cartilage regeneration, Biomaterials 127 (2017) 132-140.
[47] H. Wang, D. Zhu, A. Paul, L. Cai, A. Enejder, F. Yang, S.C. Heilshorn, Covalently Adaptable Elastin-Like Protein-Hyaluronic Acid (ELP-HA) Hybrid Hydrogels with Secondary Thermoresponsive Crosslinking for Injectable Stem Cell Delivery, Advanced Functional Materials 27(28) (2017) 1605609.
[48] N. Gjorevski, N. Sachs, A. Manfrin, S. Giger, M.E. Bragina, P. Ordonez-Moran, H. Clevers, M.P. Lutolf, Designer matrices for intestinal stem cell and organoid culture, Nature 539(7630) (2016) 560-564.
[49] F. Weeber, S.N. Ooft, K.K. Dijkstra, E.E. Voest, Tumor Organoids as a Pre-clinical Cancer Model for Drug Discovery, Cell Chem Biol 24(9) (2017) 1092-1100.

**Figure 1.** Twin reactive pairs for hydrogel formation and functionalization. **a**) Spontaneous amide bond formation by SpyTag/SpyCatcher or SnoopTag/SnoopCatcher. **b**) Hydrogel formation and functionalization with ligand of interest. ELP = elastin-like polypeptide. **c**) Central sequence in TriCatchers containing integrin-binding RGDSP (underlined) or MMP-cleavable linker (bold).

**Figure 2.** Isopeptide bond-dependent hydrogel formation, coupling of ligands and rheological analysis. **a**) Visualizing hydrogel formation. Hydrogels were formed from TriCatcher and HA-SpyTag at high concentration (each 80 µM) in PBS. TriCatcher-RGDSP (top panel) or TriCatcher-RGDSP blocked with free SpyTag peptide (bottom panel) was mixed with HA-SpyTag, allowed to gel for 30 min, and imaged after inversion. Free SpyTag peptide prevented hydrogel formation. **b**) TriCatcher functionalization. TriCatcher-RGDSP was incubated with SnoopTagJr-MBP at a molar ratio of 2:1 for 12 h, prior to boiling and SDS-PAGE with Coomassie staining. **c-e**) Rheological characterization of the hydrogel. Hydrogels were formed from TriCatcher-RGDSP and HA-SpyTag at high concentration (each 80 µM) or low concentration (each 53 µM) in growth medium. SnoopTagJr-MBP was reacted with TriCatcher-RGDSP (RGDSP + MBP) prior to hydrogel formation to a final concentration of 10.7 µM. **c**) Time-course of storage modulus (G') and loss modulus (G''). **d**) Strain sweep at 1 Hz frequency. **e**) Frequency sweep using 1% deformation.

**Figure 3.** Hydrogel erosion and swelling. Hydrogels were formed from TriCatcher and HA-SpyTag at high concentration (each 80 µM) or low concentration (each 53 µM) in PBS or growth medium. **c**) Erosion over time. The fraction of protein eroded into PBS was determined by BCA assay. (Mean ± 1 s.d., n = 4-6). **d**) Hydrogel swelling. Mass of hydrogel was determined before and after overnight incubation. (Mean ± 1 s.d., n = 4-5). **e**) TriCatcher-RGDSP hydrogel prepared at high concentration, before and after overnight incubation in PBS.

**Figure 4.** Cell behavior in the hydrogel. Mouse 3T3 fibroblasts were encapsulated in hydrogels containing varying TriCatcher components with HA-SpyTag at 80 µM each, cultured for 1 d, and analyzed by confocal microscopy showing a single z-section through the hydrogel. The brightfield image (grayscale) is shown on the left. The calcein AM ester viability stain (green) and propidium iodide dead staining (red) images are overlaid in the central panel. On the right, brightfield, calcein AM ester and propidium iodide images are overlaid. Scale bar 50 µm.

**Figure 5.** Hydrogel covalent functionalization to mimic cell-cell adhesion. **a**) MCF 10A cell spheres were grown for 6 d in low concentration TriCatcher-ELP and HA-SpyTag hydrogels functionalized with the indicated ligand and imaged by confocal microscopy. Brightfield images (grayscale) and Hoechst 33342 nuclear staining (green) are overlaid from a z-section, showing four representative fields-of-view per condition. Spheres are dissociated in the first and third image for EpCAM-SnoopTagJr and the first image for EpCAM in solution. Scale bar 50 µm. **b**) Quantification of sphere disassembly with different hydrogel functionalization (mean ± 1 s.d.). **c**) Size of cell spheres after 6 d in hydrogels functionalized by different ligands. Each dot represents one sphere (mean is shown ± 1 s.d.). *** $p<0.001$, **** $p<0.0001$, ns = not significant.