Supplementary Information

A combined SPS/LCD sensor for screening protease specificity

Louise S. Birchall, Rein V. Ulijn* and Simon J. Webb*

S1: LC observations

S1.1 Instrumentation

Unless otherwise stated, reagents, enzymes and solvents were obtained from commercial suppliers. Optical microscopy was carried out using a Zeiss Imager A1 microscope and the LC wells were viewed through crossed polarisers. Images were captured using a Canon Powershot G6 camera. Spin coating was carried out on a Laurell Model WS-400B-6NPP/LITE Spin coater (1000 rpm, 15 s).

S1.2 Liquid Crystal (LC) Chamber Preparation

S1.2.1 *Production of epoxy-functionalised glass/quartz slides*

Glass microscope slides were cut to the desired size (1.5 × 1.7 cm), immersed in piranha solution (70:30 v/v 98% H_2SO_4: 30% w/v H_2O_2) for ~ 1 h, (**WARNING: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store the solution in closed containers**), rinsed with distilled water and oven dried at 110 ºC overnight. The slides were then suspended in 3-glycidoxypropyl-trimethoxysilane and incubated at 37ºC for 1 h. This was followed by washing with e thanol and drying under a stream of N_2. The slides were used immediately.

S1.2.2 *Production of PEGA-coated slides*

A solution of monomers [mono- and bis-acrylamido polyethylene glycol (PEG), MW 1900 g mol^{-1}] (0.5 g), dimethylacrylamide (0.5 g) and 2.0 weight % photo-initiator (Irgacure) (0.02 g) in dimethylformamide (1.5 mL) was prepared and mixed for at least 6 h in the dark; the solution was kept in the dark until required. Epoxy functionalised glass slides (Genetix Ltd, or prepared as described in S1.2.1) were cut to the desired size (1.5 × 1.7 cm) using a glass cutter, washed with ethanol and dried under a stream of N_2. PEGA solution was then spin coated onto the slides to form a thin film, and a polymer sheet placed on top of the coated slide to prevent the film from drying out. Polymerisation was then carried out by placing the slide under UV radiation (365 nm) for 60 s. The polymer sheet was then removed and the slide stored under dimethylformamide until required.

S1.2.3 *Solid phase synthesis (SPS) on PEGA-coated slides*

A solution of Fmoc-protected amino acid (0.2 mM), HOBt (54 mg, 0.4 mM) and DIC (60 µL, 0.4 mM) in dimethylformamide (10 mL) was prepared. The PEGA coated slide was then covered with this solution (2 mL) and allowed to react overnight. The slide was then washed with ethanol, methanol and dimethylformamide to remove unreacted reagent. To remove the Fmoc protecting groups the slide was covered in 10% piperidine in dimethylformamide (2 mL) and left for ~45 min, followed by washing with ethanol, methanol and dimethylformamide. Peptide sequences were built up by iteratively repeating these steps, but the last Fmoc-amino acid to be coupled onto the sequences was left Fmoc-protected. In order to deprotect residue side chains, the slide was washed with ethanol, 25% aqueous ethanol and water, then covered in 1:1 trifluoroacetic acid:water (2 mL) for 60 min. The slide was then washed in dimethylformamide, methanol, ethanol and water. The Fmoc-peptide PEGA-coated slides were stored in water until required.

S1.2.4 *Production of octadecyltrichlorosilane-coated glass microscope coverslips*

Glass microscope coverslips (13 mm diameter) were cleaned with piranha solution (as described above), rinsed with distilled water and oven dried at 110 ºC overnight. They were then rinsed with absolute ethanol, dried under a stream of N_2 and heated for 2 h, before immersion in an octadecyltrichlorosilane (OTS) solution (0.5 mM in dry heptane) for 30 min. The coverslips were then rinsed with dichloromethane and dried under a stream of N_2.

S1.2.5 *Construction of LC sensor chambers*

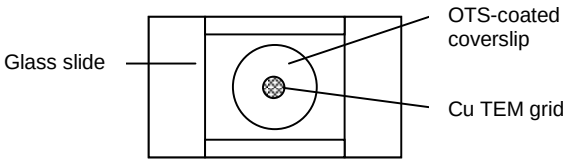

Figure S1. Diagrammatic representation of our sensor chamber.

Chambers were constructed from glass microscope slides so that the internal volume was 0.25 cm^3. OTS-coated microscope coverslips were fixed to the bottom of the chamber. Copper TEM grids (diameter 3.05 mm, well width 208 µm) were cleaned sequentially with dichloromethane, ethanol and methanol, dried under a stream of N_2, then oven dried at 110 ºC overnight. A TEM grid was placed on the OTS-coated slide, followed by the addition of 5CB (1 µL); excess 5CB was removed by contacting with a capillary tube. Any grid containing an uneven coating of 5CB was discarded. The Fmoc-peptide functionalised PEGA-coated glass slide was then placed on top of the chamber, and the appropriate aqueous solution added to the chamber. Enzyme solutions were prepared in pH 7.4 buffer [1 mg trypsin tablet (10.5 U/mg), 2.96 mg elastase (4.13 u/mgP or 3.5 U/mg), 3.5 mg thermolysin (36.5 U/mg), dissolved in MOPS buffer (1 mL, 20 mM MOPS, 100 mM NaCl) and further diluted 10-fold with buffer]. The chambers were incubated at 25 ℃. Observations were carried out every hour for a period of 10 h, then again after incubation for 24 h. Each combination of enzyme and Fmoc-peptide sequence was replicated at least three times.

S1.2.6 *Quantitative analysis of changes in LC well darkness by pixel counting*

Four wells were chosen from each grid to represent the grid as a whole, shown outlined in red in Figure S2.

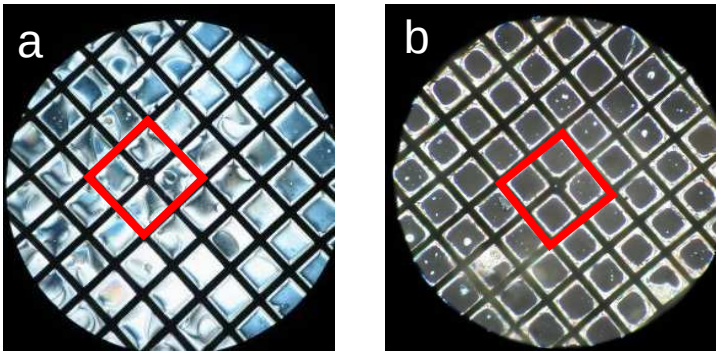

Figure S2. a) Optical appearance of the TEM grid within a chamber containing Fmoc-Phe-Phe-Lys-PEGA at 0 h. b) Optical appearance of the whole TEM grid within a chamber containing Fmoc-Phe-Phe-Lys-PEGA after 24 h incubation with trypsin.

Pixel counting was carried out using Image J software.[1] Each well of the selected four was analysed over its entire surface and a histogram created of the number of pixels at each brightness level (256 levels, Figure S3). The lower 128 levels were designated as “dark” and the upper 128 levels were designated as “light”. The number of pixels designated as “dark” was calculated before and after 24 h incubation with protease and expressed as a fraction of the total number of pixels in the well. This fraction was normalised against the maximum possible response we observed during the exposure of 5CB LC wells to surfactant; exposure to 10 mM sodium dodecyl sulphate solution for 20 min was sufficient to turn all wells uniformly dark, except at contact points with the copper TEM grid.[2]

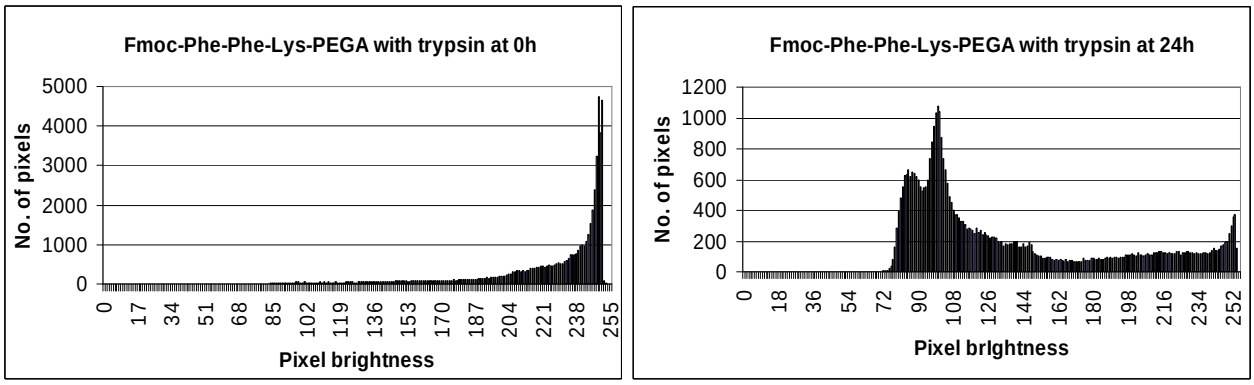

Figure S3. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Phe-Lys-PEGA both before and after incubation with trypsin for 24 h.

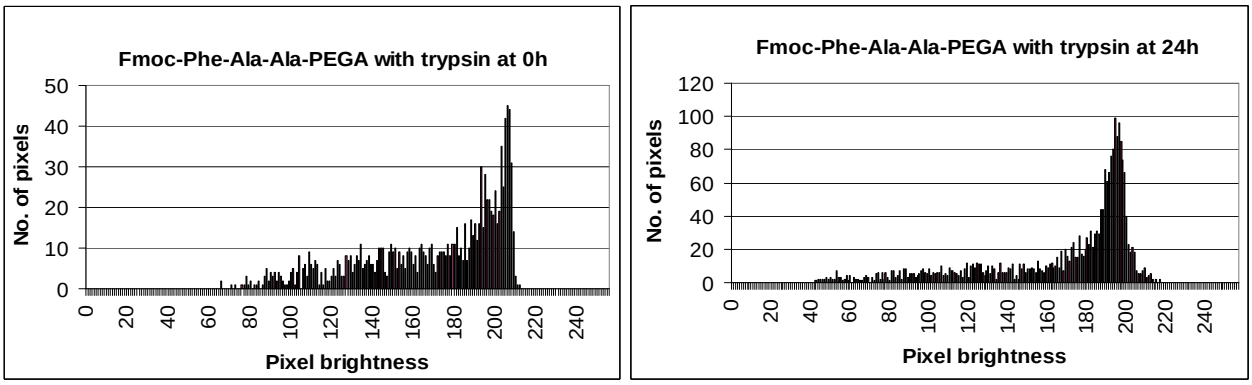

Figure S4. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Ala-Ala-PEGA both before and after incubation with trypsin for 24 h

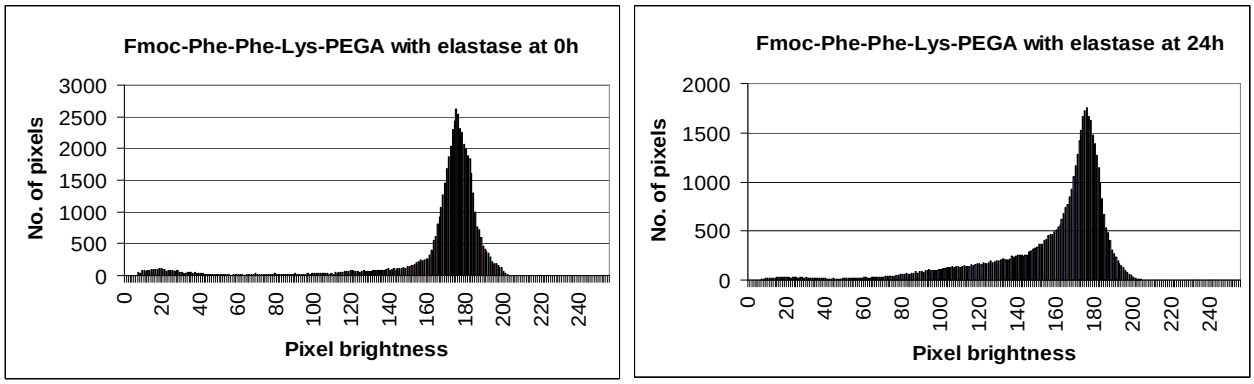

Figure S5. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Phe-Lys-PEGA both before and after incubation with elastase for 24 h.

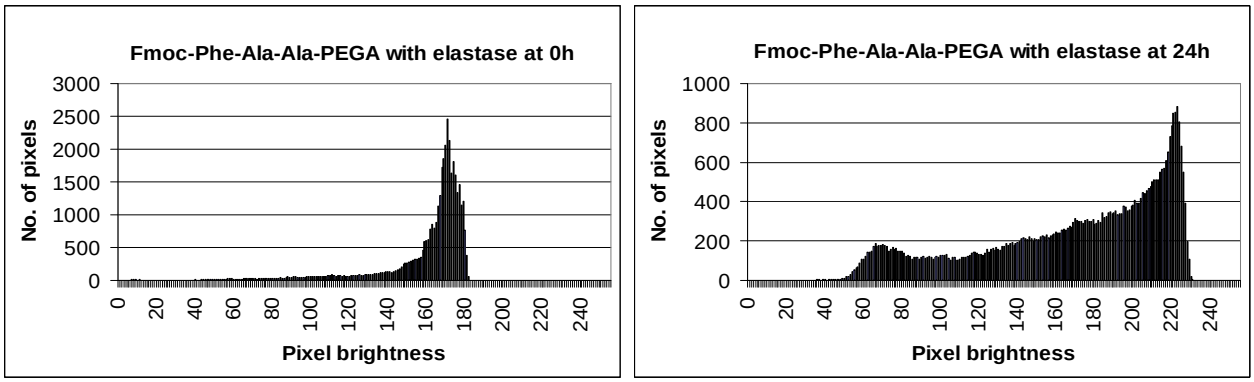

Figure S6. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Ala-Ala-PEGA both before and after incubation with elastase for 24 h.

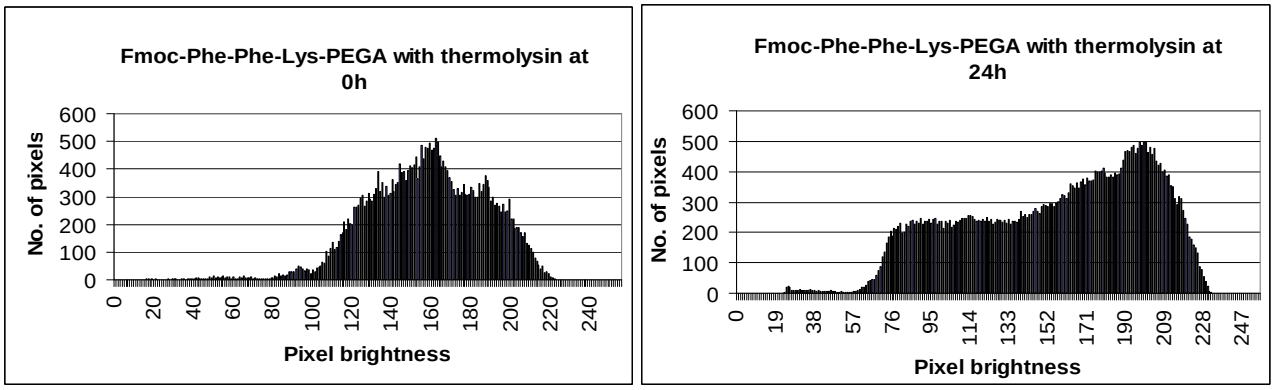

Figure S7. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Phe-Lys-PEGA both before and after incubation with thermolysin for 24 h.

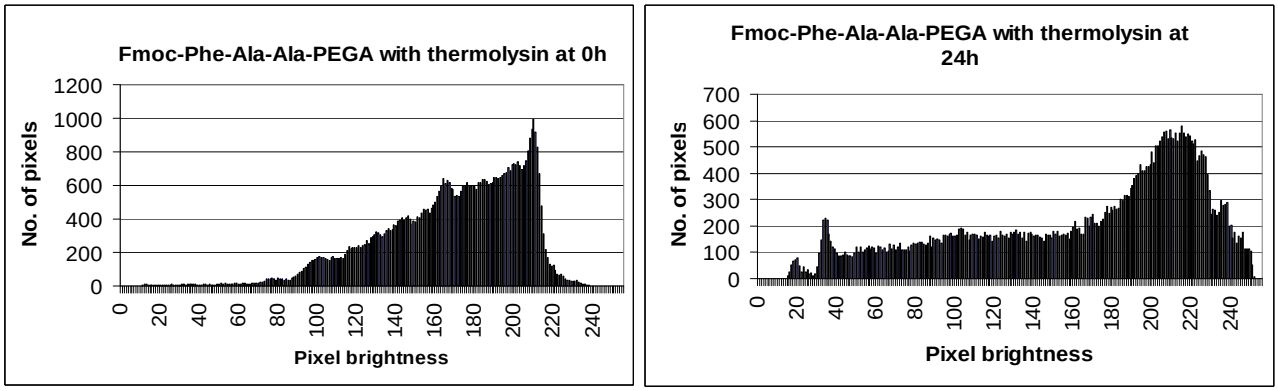

Figure S8. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Ala-Ala-PEGA both before and after incubation with thermolysin for 24 h.

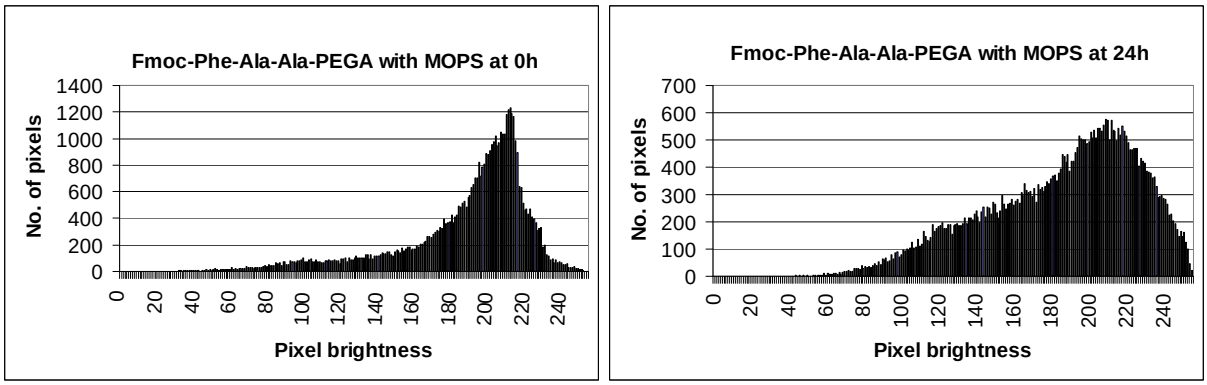

Figure S9. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Ala-Ala-PEGA both before and after 24 h incubation with MOPS buffer in the absence of added enzyme. Before incubation, 6% of pixels were dark; after incubation for 24 h with MOPS buffer in the absence of enzyme, 6% of pixels were dark.

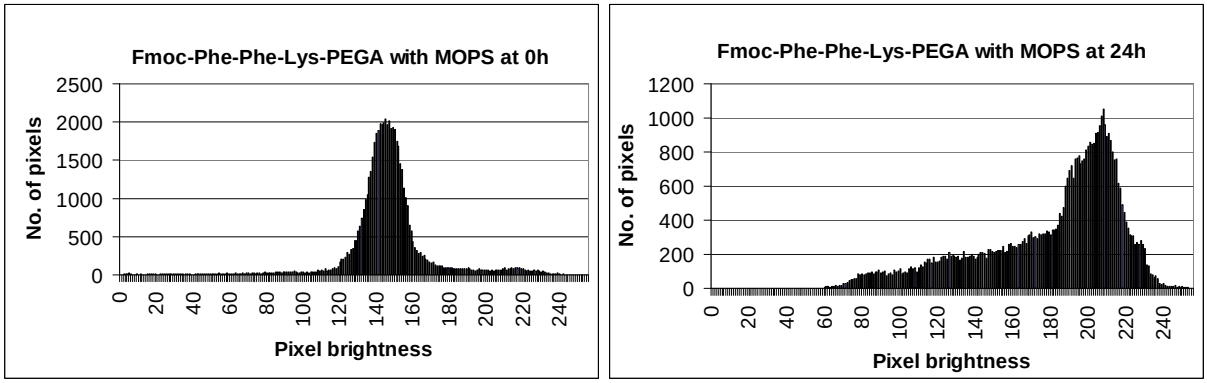

Figure S10. Histograms of pixel brightness in LC chambers containing Fmoc-Phe-Phe-Lys-PEGA both before and after 24 h incubation with MOPS buffer in the absence of added enzyme. Before incubation, 5% of pixels were dark; after incubation for 24 h with MOPS buffer in the absence of enzyme, 7% of pixels were dark.

S2: Monitoring Protease Activity by UV-Visible spectroscopy

S2.1 Instrumentation

UV-visible spectroscopy was carried out at 25 ℃ on a Jasco V-660 Spectrophotometer fitted with a thin film sample holder. PEGA-coated quartz slides, used in the place of PEGA-coated glass slides, were produced as detailed in S1.2.2. SPS was carried out on the PEGA-coated quartz slides as per S1.2.3.

S2.2 UV-Visible spectroscopy on Fmoc-peptide functionalised PEGA-coated quartz slides during SPS and after treatment with protease

Solutions with varying concentrations of Fmoc-Phe were prepared, their absorbance at 295 nm measured and the molar extinction co-efficient calculated from the calibration curve, giving $\varepsilon = 4529$ mol^{-1} dm^3 cm^{-1}. Fmoc-peptide functionalised PEGA-coated quartz slides were prepared as in S2.1. Between each coupling and deprotection step the loading of Fmoc-groups was quantified by measuring the absorbance at 301 nm (a slight shift in the absorption maximum was observed when the Fmoc peptide was attached to PEGA hydrogel), which verified that each SPS coupling and deprotection step proceeded to completion (>95%). The slides were treated for 24 h with each of the protease solutions at the concentrations detailed in S1.2.5. After incubation with enzyme solution, the Fmoc loading was determined and compared to the loading prior to enzyme treatment. Each synthesis and digest was replicated three times.

S3: Determination of the peptide fragments produced after protease incubation

S3.1 Instrumentation

S3.1.1 *Reverse-phase High Performance Liquid Chromatography*

HPLC was carried out on a Dionex P680 HPLC system fitted with a UVD170U detector. An aliquot of sample (100 µL) was injected into a Macherey-Nagel C18 column of the following dimensions: length 250 mm; internal diameter 4.6 mm; particle size 5 µm; flow rate, 1 mL min^{-1}. The gradient used was a linear exchange between 20% acetonitrile in water at 5 min to 80% acetonitrile/water at 35 min.

S3.1.2 *Reverse-phase Liquid Chromatography Mass Spectrometry*

All analyses were carried out on a reverse-phase Luna C18(2), 250 x 2mm, 5 micron column (Phenomenex). The LC-MS instrument was an Agilent 1100 Series HPLC, coupled to an Agilent 1956B Mass Detector. The gradient in Table S1 was used in all analyses; the flow rate was set at 0.5 mL min^{-1}. Mass detection was set to analyse in SCAN mode with electrospray ionisation.

Time/min	% acetonitrile + 0.1% TFA	% water + 0.1% TFA
0	10	90
5	50	50
14	85	15
20	85	15
22	10	90
25	10	90

Table S1: Solvent gradient used for the LC-MS elution of peptide fragments

S3.2 Solid phase synthesis (SPS) on PEGA beads

A solution of Fmoc-protected amino acid (1 mM), HBTU (379 mg, 1 mM) and DIPEA (230 µL, 1.3 mM) in dry dimethylformamide (6 mL) was prepared. The solution was then added to $PEGA_{1900}$ beads (0.5 g) and the mixture mixed on a blood rotor overnight. The beads were washed with ethanol, the extent of coupling determined using the Kaiser test, then the beads washed with dimethylformamide. To remove the Fmoc group the beads were covered with 20% piperidine in dimethylformamide (6 mL) and left for ~2 h. The beads were then washed with ethanol, the extent of deprotection assessed with the Kaiser test, then washed with dimethylformamide. Peptide sequences were built up by repeating these steps, however at the last step the

coupled Fmoc- amino acid was left Fmoc-protected. In order to deprotect the side chains, the beads were washed with ethanol, 25% aqueous ethanol, and water, then covered in 95% trifluoroacetic acid:water (6 mL) for 1 h. After washing with ethanol and water, the samples were stored in water until required.

S3.3 HPLC and LCMS analysis of Fmoc-peptide-PEGA bead conjugates after incubation with protease

Fmoc-peptide-PEGA bead conjugates (50 mg) were incubated with enzyme solution (1 mg of thermolysin and elastase, 1 mg trypsin tablet dissolved in 1 mL deionised water) for 2 h. The digest was drained and the beads washed with 50:50 water:acetonitrile with 0.1% trifluoroacetic acid (4 × 1 mL). For HPLC analysis, the digest and washes were combined, an aliquot (1350 µL) mixed with an internal standard (Fmoc-Arg, 250 µM, 150 µL), and the sample analysed by HPLC. Fragments in the HPLC trace were identified by comparison with known retention times for Fmoc-peptides, and quantified by comparing concentrations with the internal spike (Fmoc-Arg). For LC-MS analysis, the digests and washes were evaporated to dryness, then re-suspended in 50:50 water:acetonitrile with 0.1% trifluoroacetic acid (1.5 mL).

Sequence	**Fragments identified after incubation with trypsin**	**Fragments identified after incubation with elastase**	**Fragments identified after incubation with thermolysin**
Fmoc-Phe-Phe-Lys-PEGA Maximum concentration of Fmoc-peptides = 79 µM (after 24 hours incubation)	Fmoc-Phe-Phe-Lys-OH (**23 µM**) Fmoc-Phe-Phe-OH (**12 µM**) Fmoc-Phe-OH (2 µM)	Fmoc-Phe-OH (2 µM)	Fmoc-Phe-OH (**75 µM**)
Fmoc-Phe-Ala-Ala-PEGA Maximum concentration of Fmoc-peptides = 38 µM (after 24 hours incubation)	Fmoc-Phe-Ala-Ala-OH (<1 µM) Fmoc-Phe-OH (<1 µM)	Fmoc-Phe-Ala-Ala-OH (**15 µM**) Fmoc-Phe-Ala-OH (<1 µM) Fmoc-Phe-OH (5 µM)	Fmoc-Phe-OH (**31 µM**) Fmoc-Phe-Ala-OH (<1 µM) Fmoc-Phe-Ala-Ala-OH (<1 µM)

Table S2: Peptide fragments identified by HPLC and LCMS after incubation of peptide-PEGA bead conjugates with proteases for 2 h. Major fragments are indicated in bold-face type.

References

[S1] Available from the National Institutes of Health (http://rsb.info.nih.gov/ij/).
[S2] J. M. Brake, A. D. Mezera and N. L. Abbott, *Langmuir*, 2003, **19**, 6436-6442.