Supplementary Material (ESI) for Chemical Communications
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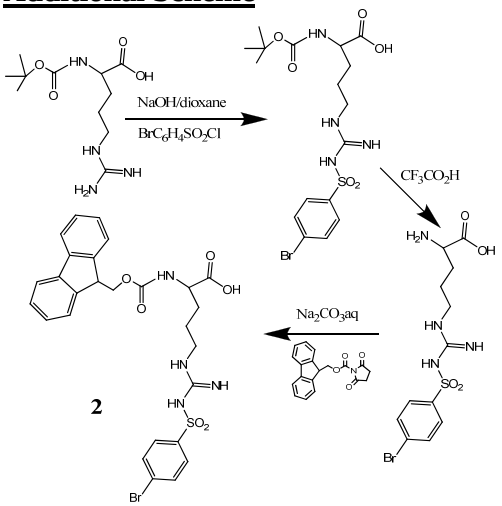
ESI

Cell adhesive hydrogels synthesized by copolymerization of arg-protected Gly-Arg-Gly-Asp-Ser methacrylate monomers and enzymatic deprotection - Perlin, MacNeil, Rimmer

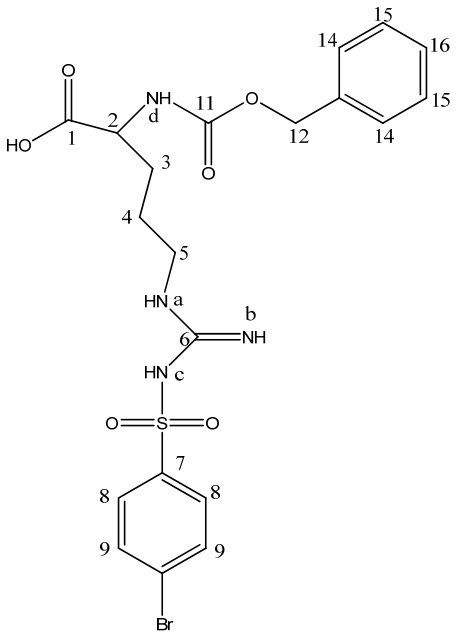
Additional Scheme



Scheme S1 Synthetic route to an orthogonally protected Arg suitable for solid phase peptide synthesis

Synthesis

Synthesis of 1



Supplementary Material (ESI) for Chemical Communications

In a typical reaction N-Carbobenzoxy-L-Arginine (Z-Arg, 5.04g) was dissolved in NaOH(aq) (3 mol.dm^{-3} , 15ml) and acetone (75ml) and then cooled to 0°C. 4-bromobenzylsulphonylchloride (12.22g) was dissolved in acetone (50ml) and added drop wise. The reaction continued for 2 hrs at 0°C followed by 2 hrs at RT during which time the pH was monitored and kept at pH 9 or above. The final reaction mixture was acidified to ~pH 6.5 with saturated citric acid solution and the acetone removed by rotary evaporation. Deionised water (10ml /g) was added and the solution further acidified to ~pH3 before the product was extracted into ethyl acetate. The organic fractions were combined, washed with water and brine and then reduced in volume to approx. 15ml. The product was precipitated into ice cold diethyl ether and then left to solidify at -8°C before the solvent was decanted off and the final traces of solvent removed under high vacuum. 40-50% yield).

^{1}H NMR: δ 1.55 (2H, m, -C^{4}**H$_2$**-), 1.80 (2H, m, -C^{3}**H$_2$**-), 2.15 (2H, m, -C^{5}**H$_2$**-), 3.20 (2H, broad s, -N^{b}**H**- / -N^{a}**H**-) 3.35 (1H, broad s, -N^{d}**H**-), 3.75 (1H, broad s, -N^{c}**H**-), 4.15 (1H, m, -C^{2}**H**-), 5.10 (2H, m, -C^{12}**H$_2$**-), 7.20-7.40 (5H, m, aromatic H's), 7.65-7.80 (4H, m, aromatic H's).

^{13}C NMR: δ 27.1 (C^{4}), 29.9 (C^{3}), 36.9 (C^{5}), 40.7 (C^{2}), 110.6 (C^{12}), 120.7 – 129.9 (aromatic C's, C$^{7\text{-}10}$ + C$^{13\text{-}16}$), 144.2 (C^{11}), 157.3 (C^{6}), 174.5 (C^{1}).

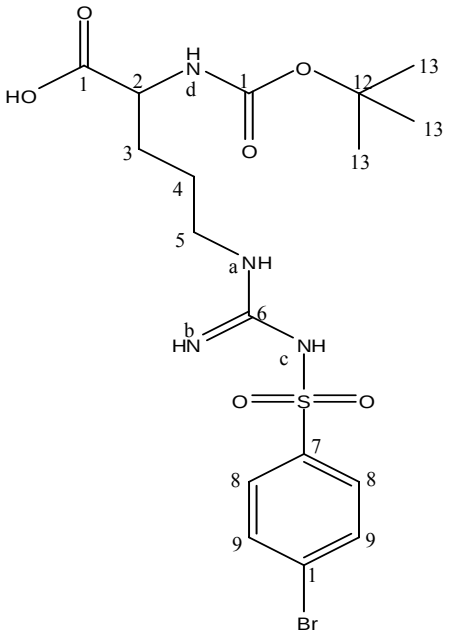
Elemental Analysis; C: 42.74, H: 4.97, N: 9.36, Br: 15.44, S: 6.46 (expected C: 45.6, H: 4.18, N: 10.6, Br: 15.20, S: 6.08).

HPLC-MS ES^{+}: elution time 16.53 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass peaks; 530, M(^{81}Br^{13}C)H^{+} (25%); 529, M(^{81}Br)H^{+} (100%); 528, M(^{79}Br^{13}C)H^{+} (23%); 527, M(^{79}Br)H^{+} (92%).

Supplementary Material (ESI) for Chemical Communications

Synthesis of 2

Step 1 Synthesis of Boc-Arg(4-Bbs)



In a typical reaction N-Boc -L-Arginine (5.01g) was dissolved in $NaOH_{aq}$ (3 mol dm^{-3} , 25ml) and dioxane (50ml) and then cooled to 0°C. 4-bromobenzylsulphonylchloride (11.01g) was dissolved in 1,4-dioxane (25ml) and added drop wise. The reaction continued for 2 hrs at 0°C followed by 2 hrs at RT during which time the pH was monitored and kept at pH 9 or above. The final reaction mixture was acidified to ~pH 6.5 with saturated citric acid solution and the solvent removed by rotary evaporation. Deionised water (50ml) was added and the solution further acidified to ~pH3 before the product was extracted into ethyl acetate. The organic fractions were combined and the product was extracted in 5% Na_2CO_3. The aqueous fractions were combined, acidified to pH 3 and then the product was extracted back into ethyl acetate. The ethyl acetate fractions were combined, reduced to ~5% of the original volume and the product was precipitated into diethyl ether. The final white solid was filtered and dried under vacuum.
^{1}H NMR: δ 1.45 (9H, s, -C^{13}**H$_3$**), 1.50-1.90 (4H, m, -C^3**H$_2$**- / -C^4**H$_2$**-), 2.05 (2H, m, -C^5**H$_2$**-), 3.15 (2H, broad s, -N^b**H**- / -N^a**H**-) 3.35 (1H, broad s, -N^d**H**-), 3.75 (1H, broad s, -N^c**H**-), 4.10 (1H, m, -C^2**H**-), 7.55-7.80 (4H, m, aromatic H's).
^{13}C NMR: δ 26.6 (C^4), 28.7 (C^{13}), 30.1 (C^3), 48.0 (C^5), 57.8 (C^2), 81.2 (C^{12}), 127.8 (C^{10}), 128.9 (C^8), 132.8 (C^7), 133.0 (C^9), 155.2 (C^{11}), 165.0 (C^6), 175.9 (C^1).
Elemental Analysis; C: 38.44, H: 4.81, N: 10.97, Br: 15.73, S: 6.46 (expected C: 41.38, H: 5.11, N: 11.36, Br: 16.20, S: 6.50).
HPLC-MS ES$^+$: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass

peaks; 496.3, M($^{81}Br^{13}C)H^+$ (17%); 495.3, M($^{81}Br)H^+$ (95%); 494.3 M($^{79}Br^{13}C)H^+$ (23%); 493.3, M($^{79}Br)H^+$ (100%).

Step 2 Removal of Boc from Boc-Arg(4-Bbs) with TFA

Boc-Arg(4-Bbs) was dissolved in TFA (20ml/g) and allowed to react at room temperature for 2 hours. The volume of liquid was reduced to ~10% by distillation under reduced pressure and the product was precipitated into diethyl ether. H-Arg(4-Bbs) was triturated with 3 x 100ml diethyl ether to remove traces of TFA. A typical reaction used 3.0981g of Boc-Arg(4-Bbs) and 60ml of TFA.

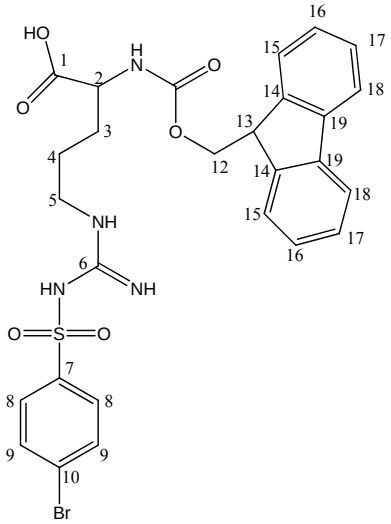
^{1}H NMR: δ 1.15 (2H, t, -C^3**H$_2$**-, J=7.5Hz), 1.65 (2H, s, -C^4**H$_2$**-), 1.95 (2H, s, -C^5**H$_2$**-), 2.95 (2H, broad s, -N^b**H**- / -N^a**H**-) 3.15 (1H, broad s, -N^d**H**-), 3.55 (1H, broad s, -N^c**H**-), 4.05 (1H, m, -C^2**H**-), 7.85-8.05 (4H, m, aromatic H's).

^{13}C NMR: δ 26.6 (C^4), 27.3 (C^3), 48.5 (C^5), 51.7 (C^2), 125.5 (C^{10}), 127.7 (C^8), 128.7 (C^7), 131.2 (C^9), 156.7 (C^6), 170.9 (C^1).

Elemental Analysis; C: 34.51, H: 4.36, N: 13.11, Br: 20.54, S: 8.01 (expected C: 36.65 , H: 4.36, N: 14.25, Br: 20.32, S: 8.15).

HPLC-MS ES$^+$: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm), mass peaks; 496.3, M($^{81}Br^{13}C)H^+$ (17%); 495.3, M($^{81}Br)H^+$ (95%); 494.3 M($^{79}Br^{13}C)H^+$ (23%); 493.3, M($^{79}Br)H^+$ (100%).

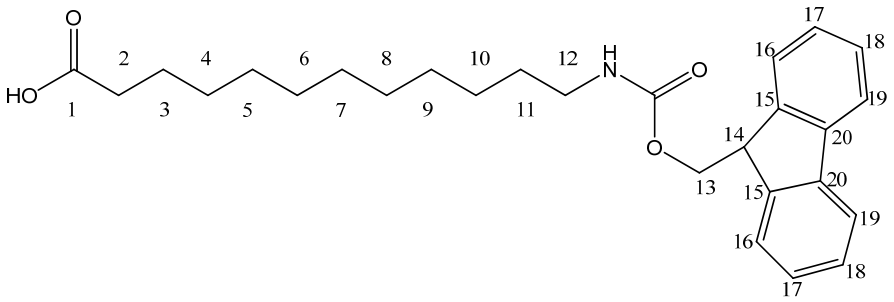
Step 3 Addition of FMOC



H-Arg(4-Bbs)-OH (2.49g, 5.65mmol) was dissolved in 6% aqueous sodium carbonate (21ml) and the solution was cooled to 0°C. A solution of 9-fluorenylmethyl succinimidyl carbonate (fmoc-Cl),(1.92g, 5.66mmol) in dioxane (10ml) was added drop wise and the reaction left to stir for 1h with the ice bath removed. The solution was diluted with water (100ml) and washed with ether (2 X 50ml) before acidification with saturated citric acid solution (30ml). The solution was extracted with ethyl acetate (3 X 100ml) and the combined extracts were washed with water (X 2), before drying over $MgSO_4$. The dried solution was concentrated in vacuo and the desired product precipitated by the addition of diethyl ether (3.3g, 89%).

^{1}H NMR: δ 1.30 (2H, m, -C^{4}**H$_2$**-), 1.60 (2H, m, -C^{3}**H$_2$**-), 2.15 (2H, m, -C^{5}**H$_2$**-), 3.25 (2H, broad s, -N^{b}**H**- / -N^{a}**H**-) 3.30 (1H, broad s, -N^{d}**H**-), 3.70 (1H, broad s, -N^{c}**H**-), 4.05 (1H, m, -C^{2}**H**-), 4.20 (1H, m, -C^{13}**H$_2$**-), 4.35 (2H, d, $J_{C12\text{-}C13}$=6.9Hz, -C^{12}**H$_2$**-), 7.20-7.40 (6H, m, aromatic H's), 7.65-7.80 (4H, m, aromatic H's), 7.95 (2H, d, aromatic H's).
^{13}C NMR: δ 29.9 (C^{4}), 31.5 (C^{3}), 45.3(C^{5}), 55.0 (C^{2}), 67.7 (C^{12}), 127.1 – 133.0 (aromatic C's, C$^{7\text{-}10}$ + C$^{13\text{-}19}$), 144.2 (C^{11}), 157.3 (C^{6}), 174.5 (C^{1}).
Elemental Analysis; C: 51.86, H: 4.39, N: 9.03, Br: 14.81, S: 6.10 (expected C: 52.69, H: 4.42, N: 9.10, Br: 15.73, S: 6.46).
HPLC-MS ES^{+}: elution time 15.60 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 619.2, M(^{81}Br^{13}C$_2$)H^{+} (9%); 618.2, M(^{81}Br^{13}C)H^{+} (32%); 617.2, M(^{81}Br)H^{+} (100%); 616.2 M(^{79}Br^{13}C)H^{+} (29%); 615.2, M(^{79}Br)H^{+} (86%).

Synthesis of Fmoc-ADDA



In a typical reaction12-aminododecanoic acid (ADDA) (5.07g) was suspended in Na_2CO_{3aq} (10%, 125 ml) solution and cooled to 0ºC. Fluorenylmethoxy carbonyl chloride (6.15g) in dioxane (50ml) was added. The reaction was allowed to warm up to room temperature and stirred for 4 hours. The dioxane was removed by rotary evaporation and water (250ml) was added. The solution was acidified to pH 3 by addition of concentrated HCl. The product (Fmoc-ADDA) was extracted into ethyl acetate and then dried with magnesium sulphate. The solvent was removed to give the crude product. This was re-crystallised from acetonitrile to form the pure product.
^{1}H NMR: δ 1.25-1.60 (18H, m, -C**H$_2$**-, C$^{3\text{-}11}$ alkyl H's), 2.15 (2H, s, -C^{2}**H$_2$**-), 2.35 (2H, t, $J_{C11\text{-}C12}$=6.25Hz, -C^{12}**H$_2$**-), , 3.20 (1H, m, -N^{a}**H**-), 4.20 (1H, m, -C^{15}**H$_2$**-), 4.35 (2H, d, $J_{C15\text{-}C14}$=6.25Hz, -C^{14}**H$_2$**-), 7.25-7.80 (8H, m, aromatic H's).
^{13}C NMR: δ 24.9-34.0 (C$^{2\text{-}11}$ alkyl C's), 41.9 (C^{15}), 47.2 (C^{12}), 68.1 (C^{14}), 119.9 – 127.6 (aromatic C's, C$^{7\text{-}10}$ + C$^{13\text{-}19}$), 141.3 (C^{13}), 145.3(C^{1}).
Elemental Analysis; C: 72.18, H: 8.29, N: 2.96 (expected C: 74.47, H: 8.26, N: 3.10).
HPLC-MS ES^{+}: elution time 18.3 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 452.8, MH^{+} (100%); 453.8, M(^{13}C)H^{+} (25%).

Peptide synthesis
Peptides were synthesised using a Wang resin with a nominal resin loading of 1.1 mmol./g using DMF as solvent and Fmoc amino acid protection. Amino acids were

coupled to the growing sequence using the HBTU/HOBt/DIPEA procedure. Fmoc protecting groups were removed with 20% piperidine in DMF. The deprotection was assessed by use of the picryl sulfonic acid test. The final peptide was cleaved from the resin using a cleavage cocktail of 95% TFA, 2.5% water and 2.5% triisopropylsilane (TIS) (10 ml per g of resin) for 1 hour. The resin was washed with 1 x cleavage cocktail, 3 x DCM and 3 x ACN.

Analysis and Purification of Peptide Sequences

All HPLC analyses were done using a Waters 2690 separation module with a Waters 996 photodiode array detector and an Omnisphere 5 C18 column (250 x 4.6 mm) unless otherwise stated. A gradient of 95% water (containing) 0.1% trifluoroacetic acid): 5% acetonitrile to 100% acetonitrile over 25 minutes was employed. A Micromass Platform LCZ mass spectrometer was used to analyse the traces. Peptides that were determined to have a purity of less than 90% after precipitation from diethyl ether were purified by preparative reverse phase HPLC. The analytical results from the two peptides were as follows:

Peptide **3**

HPLC-MS ES^+: elution time 11.65 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 781.1, M($^{81}Br^{13}C_2$)H^+ (12%); 780.1, M($^{81}Br^{13}C$)H^+ (39%); 779.1, M(^{81}Br)H^+ (100%); 778.1 M($^{79}Br^{13}C$)H^+ (31%); 777.1, M(^{79}Br)H^+ (92%). 95% purity by integration of peaks generated by diode array.

Peptide **4**

HPLC-MS ES^+: elution time 15.67 minutes (5% ACN: 95% H_2O cont. 0.1% TFA to 100% ACN gradient over 25 minutes, Omnisphere 5 C18 column 250 x 4.6 mm). Mass peaks; 978.3, M($^{81}Br^{13}C_2$)H^+ (17%); 977.3, M($^{81}Br^{13}C$)H^+ (48%); 976.3, M(^{81}Br)H^+ (100%); 975.3 M($^{79}Br^{13}C$)H^+ (39%); 974.3, M(^{79}Br)H^+ (81%). 64% purity by integration of peaks generated by diode array before preparative HPLC, 95% purity after purification.

Determination of GST Deprotection by HPLC

All HPLC analyses were done using a Waters 2690 separation module with a Waters 996 photodiode array detector and an Omnisphere 5 C18 column (250 x 4.6mm). A gradient of 95% water (containing) 0.1% trifluoroacetic acid): 5% acetonitrile to 100% acetonitrile over 25 minutes was employed with a 25min downwards gradient included where enzyme was present to wash the enzyme from the column. The amino acids were detected using a photodiode array from 200 to 320nm. The concentration of the arginine derivative amino acid was determined by integrating the peak areas and using the ratio of peak areas to determine the concentration from a calibration chart. The calibration charts were formulated using 0, 20, 40, 60, 80, 100, 200, 300, 400 and 500μM solutions of the

amino acid with 50, 100, or 1000μM of the reference amino acid and 500mmol dm^{-3} of GSH.

Synthesis of polymers

Stock solutions of the peptides were made with methanol to a concentration of 10 picomoles / ml. These stock solutions were diluted with methanol to form solutions with concentrations of 1, 0.1 and 0.01pmol/ml. Stock solutions of GMA, EDMA and 1 wt% initiator were made up and stored at -8ºC in the dark until use. Peptide and monomer stock solutions were added and combined immediately prior to use. UV cured polymer thin-films were synthesised as described in section 5.1.2.

Enzyme Deprotection of Peptides

Disks of polymers were cut out and placed into 24 well plates in a sterile culture hood. The disks were washed three times with ethanol and three times with PBS. A stock solution of GST (from equinine liver lyophilized powder, ≥25 units/mg protein, Sigma-Aldrich) (1mg/ml) and GSH (10 mmol dm^{-3}) in PBS was made up and then filter sterilised. The stock solution was diluted by a factor of 20 with sterile PBS resulting in a final concentration of GST 0.05mg/ml and GSH 500 μM. The final enzyme-containing solution was added to wells containing polymers (0.5 ml/well) and these were placed in an incubator at 37ºC and 5% CO_2 for a set time period. After the enzyme incubations were complete the polymers were washed three times with PBS and twice with serum free media prior to cell culture.

Cell Culture

All materials were obtained from Sigma-Aldrich UK. Deionised water was used throughout. Dulbecco's modified eagles media (DMEM) supplemented with $2x10^{-3}$ mol L^{-1} glutamine, 0.625 μg ml^{-1} amphotercin B, 100 i.u. ml penicillin and 100 μg ml^{-1} streptomycin was routinely utilised for all experiments. In addition where serum containing media was utilised 10% v/v fetal calf serum was added.

Cell Maintenance

Fibroblasts were obtained from human skin obtained from patients undergoing elective surgery who gave informed consent for skin not required for their treatment to be used for research (as detailed in reference ESI 1). Skin was placed in 0.1% trypsin w/v overnight before the epidermis was peeled from the dermis. This dermis was washed in phosphate-buffered saline (pH 7.2), finely minced and then placed in 0.5% collagenase-A at 37ºC for 16 h. Cells were then cultured in DMEM media supplemented with 10% v/v neonatal calf serum, $2x10^{-3}$ mol L^{-1} glutamine, 0.625 mg ml^{-1} amphotercin B, 100 i.u. ml penicillin and 100 mg ml^{-1} streptomycin. Cells were passaged routinely using 0.02% (w/v) EDTA and used for culture on polymers between passage numbers 5 and 12.

Supplementary Material (ESI) for Chemical Communications

Cell Culture on Polymers

Cells were harvested at ~90% confluency by incubating with trypsin for 5 min . Media containing 10% serum was added to neutralise the trypsin and the cell suspension was centrifuged and the media containing trypsin was decanted. After harvesting cells were suspended in the serum free medium. The concentration of cells was determined by mixing 20µl of the cell suspension with 20µl of trypan blue and counting the number of live cells in a known volume using a haemocytometer. The cell suspension was diluted to give an appropriate volume that would contain 100,000 cells (20-40µl). This volume was added to each well containing polymer or control substances and the cells were allowed to adhere for 20 minutes. After this time 1 ml of the appropriate media was applied to the wells and the cells were incubated for a set time period after which time the cells were photographed through a phase contrast microscope and/or analysed via the MTT assay.

Competition with Soluble RGD

A solution of 1 mg/ml GRGDS in serum-free media was prepared. After 24 hours of cell culture as described above the media was removed from all wells. 0.5ml of GRGDS containing media was added to half of the wells and 0.5 ml of serum free media was applied to the other half of the wells as a control. Photographs of the cells were taken at 0, 15, 30, 45 and 60 minutes after the application of the GRGDS-containing media through a phase contrast microscope. After 60 minutes the cells were analysed by the MTT assay as described below.

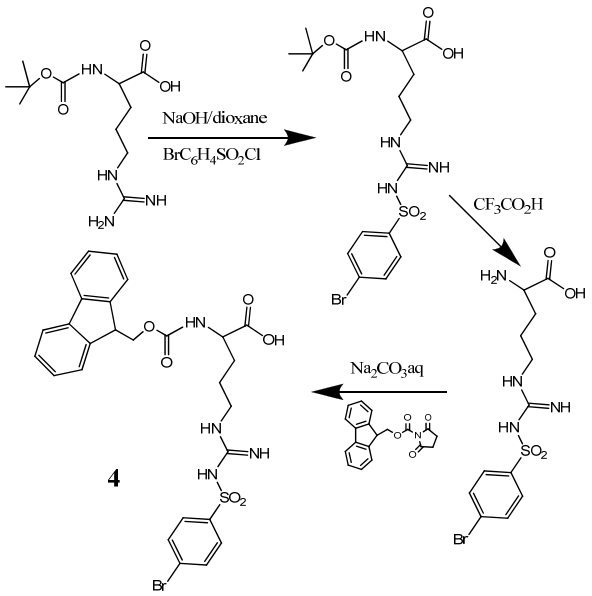
MTT Assay

MTT (Sigma-AldrichTM) acts as an artificial hydrogen acceptor substrate coloured formazan product which is then eluted from the cells using acidified isopropanol. This cytobiochemical assay provides a direct indication of the viability of cells and can be used to provide an indirect reflection of cell number. Cells were washed with PBS and then 1 ml of MTT solution was added (0.5 mg MTT / ml PBS) to each well. The cells were incubated at 37ºC and 5% CO_2 for 40 minutes. The MTT solution was removed and 300µl of ethoxy ethanol was added to each well to solubilise the formazan colour formed by the assay. 150µl of ethoxy ethanol was removed from each well and placed in a 96 well plate. The absorbance at 540 nm was read using a Dynatech MR5000 plate reader with a reference wavelength of 630nm.

Visualisation of cell cytoskeleton by staining for F actin

Cellular organization on the hydrogels was examined by staining the cells for Factin which is the major cytoskeleton component which allows cells to spread and become spatially organized on substrates.After a set time point (30mins, 1,2,4 or 24 hours) cells were washed with PBS and then fixed for 10 minutes with 10% formalin in PBS. The cells were washed with PBS x 3 and then 0.1% triton was added for 10 minutes to permeabilise the cell membranes. The cells were again washed with PBS x 3 and then 250µl of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (300nM in PBS) was added for 20 minutes. Following a further 3 PBS washes a solution of FITC-

phalloidin (0.5μg/ml in PBS containing 1% methanol) was added for 5 minutes. After a further 3 PBS washes the cells were observed visually using epi-fluorescence microscopy using an ImageXpress® automated cellular imaging and analysis system (DAPI λ_{ex} = 358 nm, λ_{em} = 461 m; FITC λ_{ex} = 490 nm, λ_{em} = 523 nm; Axon Instruments, CA, USA).



Scheme S1 Synthetic route to an orthogonally protected Arg suitable for solid phase peptide synthesis

References

ESI 1 MC Higham, R. Dawson, M Szabo, R. Short, D.B. Haddow, S MacNeil *Tiss. Eng.* 2003, **9**, 919