Supporting information to

Cell Adhesion on RGD-Displaying Knottins with Varying Number of Tryptophan Amino Acids to Tune the Affinity for Assembly on Cucurbit[8]uril Surfaces

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1. General methods

Chemicals were purchased from Sigma Aldrich or from Acros Organics unless differently specified and used without further purification. The concentration of each batch of CB[8] (Sigma Aldrich) was assessed by UV-Vis titration with cobaltocenium according to a literature procedure.[S1] Dulbecco's Phosphate Buffered Saline (PBS, Sigma Aldrich) with pH 7.4 at 25 °C containing 0.01 M phosphate buffer, 2.7 μM potassium chloride and 0.137 M sodium chloride was used. Cell staining reagents were purchased from Invitrogen. NMR spectra were recorded on a Bruker spectrometer (Ascend 400). Polymerase chain reaction (PCR) was performed using a Peqlab Primus 25 advanced thermocycler. UV–Vis measurements to determine DNA and protein concentrations were performed using a Thermo Scientific Nanodrop 1000. Matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis was performed with a Waters (Maldi Synapt) high definition mass spectrometer. UV–Vis measurements for trypsin inhibitor assays were performed using a PerkinElmer (Victor X3) multiwell plate reader. Surface plasmon resonance (SPR) experiments were conducted using SPR gold substrates (50 nm thickness of gold) from Ssens BV on a Resonant-probes SPR. The reflectivity was measured at fixed angle at which point the linear region of the SPR curve stopped. Fluorescence microscopy was performed using an Olympus (1X71) microscope with appropriate filter settings.

2. Molecular cloning of knottin constructs

The W2 knottin gene was first constructed using assembly PCR. ssDNA sequences, with complementary overhangs, corresponding to the W2 knottin flanked by 5' enterokinase cleavage site and BsrGI restriction site sequences and 3' NheI restriction site sequence were designed using the Assembly PCR Oligo Maker tool[S2] and ordered from Eurofins MWG Operon, Germany. Assembly PCR was performed using pfu DNA polymerase. This PCR product and a pET15b TFP-K0 plasmid[27] were then digested with BsrGI and NheI restriction enzymes (NEB) and mixed together in a 5:1 insert:plasmid molar ratio for ligation using T4 DNA ligase (NEB). This resulted in the plasmid pET15b-TFP-W2, which was then transformed into Novablue ultracompetent cells (Novagen). The plasmid was extracted from selected colonies and the insert was sequenced by Eurofins MWG operon and verified to be correct. Site-directed mutagenesis was performed simultaneously with 4 sets of primers to mutate GGWGG sequences to GGSGG and vice versa. The Quick change lightning multi site-directed mutagenesis kit (Agilent) was used and mutated plasmids were transformed in XL 10 Gold ultracompetent cells. Several colonies were selected, grown in culture and the plasmids extracted from them were sent for sequencing. The plasmids pET15b-TFP-W0, pET15b-TFP-W1, pET15b-TFP-W3 and pET15b-TFP-W4 were thus obtained. One more site-directed mutagenesis was performed to mutate the RGD sequence in W4 to RGE resulting in the pET15b-TFP-W4E plasmid. The six different plasmids were transformed into the expression host, Rosetta-Gami 2(DE3)pLysS competent cells (Novagen), and grown overnight on LB agar plates containing 34 mg/L chloramphenicol and 100 mg/L ampicillin. Individual colonies were grown in LB media containing the mentioned antibiotics. For long-term storage, 15%-glycerol bacterial stocks were made and placed at −80 °C. The primers used for these constructs are presented here:

W2 Assembly PCR Primers	
W2 assem f1	AGTCAGTCAGTCAGTCAGTCTGTACAATGATGATGATGATAAAGGTGAGGG
W2 assem r2	AACCTCCGCTGCTGCCACCCGACCCTCCGCTCTTGCCCTCACCTTTATCATCATCATC
W2 assem f3	GCAGCAGCGGAGGTTGGGGTGGAAGCTCCGGAAGTGGTAGCGGAGGCTGCCCG
W2 assem r4	CCTGGCTGCAGGTCAGCGGCGGGTTATCGCCGCGCGGGCGCGGGCAGCCTCCG
W2 assem f5	CTGACCTGCAGCCAGGATAGCGATTGCCTGGCGGGCTGCGTGTGCGGCCCGAAC
W2 assem r6	CAACCACCGCTACTACCGCTTCCACTACCGCCGCAAAAGCCGTTCGGGCCGCACA
W2 assem f7	CGGTAGTAGCGGTGGTTGGGGAGGGAGCAGCGGTGGATCGGGAGGGAGCGGCGAAG
W2 assem r8	CTTGGAACCTTGGAACCTTGGCTAGCTTATTTGCCTTCGCCGCTCCCTC
Fwd	Agtcagtcagtcagtcagtc
Rev	Cttggaaccttggaaccttg
Mutagenesis primers	
S13W	Gcaagagcggagggtggggtggca
W20S	Ggcagcagcggaggttcgggtggaag
W72S	Cggtagtagcggtggttcgggagggag
S79W	Ggagcagcggtggatggggaggga
RGD>RGE	Ccgcgcggcgagaacccgccgct

DNA sequence of the W2 knottin:

agtcagtcagtcagtcagtcTGTACAatgatgatgatgataaaggtgagggcaagagcggagggtcgggtggcagcagcggaggtt
ggggtggaagctccggaagtggtagcggaggctgcccgcgcccgcgcggcgataacccgccgctgacctgcagccaggatagcgatt
gcctggcgggctgcgtgtgcggcccgaacggcttttgcggcggtagtggaagcggtagtagcggtggttggggagggagcagcggtgg
atcgggagggagcggcgaaggcaaataaGCTAGCcaaggttccaaggttccaag

Protein sequence of the W2 knottin:

DDDDKGEGKSGGSGGSSGGWGGSSGSGSGGCPRPRGDNPPLTCSQDSDCLAGCVCGPN
GFCGGSGSGSSGGWGGSSGGSGGSGEGK

3. Protein expression and purification

Five milliliters of bacterial starter cultures was grown overnight from glycerol stocks at 37 °C with shaking in LB media containing appropriate antibiotics. This was then transferred into 1 L of the same media, and the cultures were grown until they attained $O.D._{600\ nm}$ values between 0.4 and 0.8. Protein expression was then induced using isopropyl-β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. These cultures were grown overnight at 18 °C with shaking. The cultures were then spun down at 6000 rcf for 10 min at 4 °C, and supernatants were discarded. Bacterial pellets were resuspended in 10 mL of BugBuster protein extraction reagent (Novagen) with 10 μL of benzonase (30 U/μL, Novagen) and gently shaken for 20 min at 25 °C. TFP-fused knottin constructs were then purified using His-Select nickel affinity columns (Sigma-Aldrich) into an elution buffer of 50 mM NaH_2PO_2, 300 mM NaCl, and 250 mM imidazole at pH 8. The purified TFP-fused knottins were then rebuffered into pH 7.4 phosphate buffered saline (PBS, Sigma-Aldrich) using 30 kDa-cutoff centrifugal filter units (Amicon Ultra). Concentrations of these TFP-

fused knottins were determined from the absorbance value at λ = 467 nm and an extinction coefficient of 64000 M^{-1} cm^{-1}. Knottins were cleaved from the TFP using an enterokinase enzyme (EKMax, Life Technologies). The optimized reaction conditions required 50 ng of protein per 30 μL of reaction with 0.1 units of enterokinase in pH 7.5 buffer containing 20 mM trisHCl, 10 mM NaCl, and 2 mM $CaCl_2$ at 37 °C for 16 h. SDS-PAGE analysis is shown in Figure S1. The cleaved knottin was isolated from the other proteins using a 10 kDa-cutoff centrifugal filter unit (Amicon Ultra) followed by a 3 kDa-cutoff centrifugal filter unit (Amicon Ultra). Final knottin solutions were in PBS. At particular steps SDS-PAGE analysis was performed using Bio-Rad Mini-PROTEAN TGX Stain-Free Precast 4-15% Gradient Gels in conjunction with a Mini-PROTEAN Tetra Vertical Electrophoresis Cell. Protein samples were prepared by mixing them in 1:1 volume ratio with the Bio-Rad Sample preparation buffer (2X) with β-mercaptoethanol added and heated to 95°C for 10 mins. The samples were loaded in the gel and 120 V potential was applied for electrophoresis. The gels were then activated and imaged using the Gel Doc EZ Gel Documentation System and a Stain-Free filter tray.

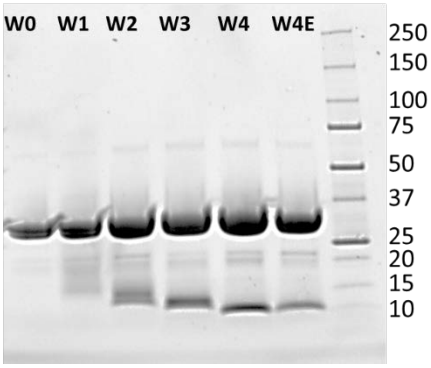

Figure S1. SDS-PAGE analysis of the enterokinase cleavage of the knottin constructs from TFP.

4. Knottin concentration determination

The concentrations of the cleaved knottin constructs were determined using UV-Vis spectroscopy. The tryptophan (W) units exhibit their mean peak absorbance at $\lambda = 280$ nm (W_{280}) and the cystines (CC) exhibit their mean peak absorbance at $\lambda = 250$ nm (CC_{250}). W also has an absorbance contribution at 250 nm (W_{250}) and CC also contributes to the absorbance at 280 nm (CC_{280}).

The contribution of W at $\lambda = 250$ nm was determined from its spectral information[S3] yielding:

$$W_{250}/W_{280} = 0.406$$

From W0, which contains no W units, the absorbance at $\lambda = 250$ nm and 280 nm arises purely due to the presence of the CC units and from this:

$$CC_{280}/CC_{250} = 0.324.$$

This value also corresponds with what was determined in literature.[S4]

These provide us with the set of linear equations:

$$CC_{250} + 0.406W_{250} = A_{250}$$

$$0.323CC_{250} + W_{280} = A_{280}$$

Where A250 and A280 are the total absorbance at 250 nm and 280 nm respectively as found in Figure 5.2d. Using these linear equations, it was possible to determine the absorbance purely due to W at 280 nm and CC at 250 nm.

Extinctions coefficients of W4, W3 and W2 at 280 nm were determined as 22375 M^{-1} cm^{-1}, 16875 M^{-1} cm^{-1} and 11375 M^{-1} cm^{-1} respectively using Expasy's Protparam tool[37] assuming all cysteines

form disulfides. These values allowed us to determine the concentrations of W4, W3 and W2 from their well-defined A_{280} absorbance peaks.

From this, the concentration:CC_{250} ratio was determined and used to calculate the concentrations of W0 and W1 from the CC_{250} values determined previously using the linear equations.

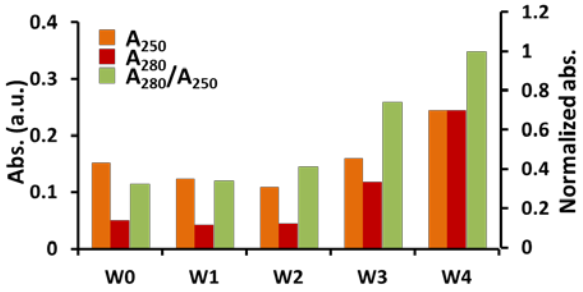

Figure S2. UV-Vis absorbance values at λ = 280 and 250 nm (left y-axis) and their ratios (right y-axis) for the cleaved and purified knottin construct solutions.

5. MALDI-ToF

For MALDI-ToF analysis, protein samples in water/buffer were mixed with equal proportions of acetonitrile + 0.1% TFA. Samples were prepared at a final protein concentration between 30 - 50 μM and this was mixed with sinapinic acid (σ)-matrix.

6. Synthesis of thiol-functionalized MV^{2+}

The synthesis of MV^{2+} was adapted from Rauwald, et al.[S5] and González-Campo, et al.[S6]

7. Surface functionalization

Gold substrates (1" glass, 20 nm Au, Sens BV) were cleaned in piranha for 20-30 s followed by thorough rinsing in Milli-Q water and drying with N_2. Substrates were immersed in a 1 mM

ethanolic solution of the disulfides (1% maleimide, v/v) overnight under argon. MV^{2+} was then reacted (200 μM, PBS pH 7.4, freshly prepared) by incubation for 1 h at room temperature. Further incubation with CB[8] or CB[8] and knottins was done for 1 h at room temperature under ambient light conditions, after which the substrates were briefly dip-rinsed in MilliQ and dried with N_2. CB[8] was 50 μM in 0.1x PBS for all conditions. For covalently attached RGD, the same procedure was followed replacing MV^{2+} with Cov.

For cell experiments knottin solutions were 2000 nM in 50 μM CB[8] for W1*-W4* at high surface density and W4E while at low surface density knottins solutions were 3600, 1000, 75 and 100 nM in 50 μM CB[8] for W1, W2, W3, and W4 respectively. Fibronectin surfaces were prepared on glass slides cleaned with piranha for 1-5 minutes, incubated with 10 g/mL fibronectin at room temperature for 1 h. then rinsed with PBS for 0.5-1 h and used directly.

8. Surface Plasmon Resonance (SPR)

SPR measurements were carried out under conditions of constant flow (50 μL/min) using a pH 7.4 buffer of 0.5 × PBS. Surfaces were prepared as described above. All knottin solutions also contained 50 μM CB[8].

9. Cell culture

C2C12 mouse myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-Glutamine, 1% v/v Penicillin/Streptomycin and 10% v/v Fetal Bovine Serum (FBS) and used between passage 10 and 30. Cells were sub-cultured on functionalized surfaces in serum depleted medium (without FBS).

Cells adhered to the surfaces (cell density 8,000-10,000 cells/cm^2) were fixed directly with cold 4% paraformaldehyde (PFA) for 10 min at room temperature (RT) and washed thrice in PBS. Cells were then permeabilized in 0.5% Triton X-100 and 5% Bovine Serum Albumin (BSA) in PBS for 10 min at RT and blocked with 0.1% Triton X-100 and 5% BSA in PBS (PBST) for 60 min at RT. Immunocytochemical labelling of cell proteins was done in PBST for 1 h at 1:200 for Vinculin-FITC and 1:100 Phalloidin 568 (Actin). Subsequently cells were washed with PBS twice and labelled with 4',6-diamidino-2-phenylindole (DAPI) 1:1000 in PBS. Immunolabelled cells were imaged using an inverted fluorescent microscope with corresponding excitation and emission filters.

10. Data analysis

Data were analyzed by using Mood's median test for non-normal distributions comparing all groups of interest (OriginPro v.2015, OriginLab). Image analysis was performed using Image J[S7] and Cell Profiler image analysis software.[S8] Quantification of cell shape factors were performed by using Cell Profiler algorithms.

11. References

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