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Selective, Non-Covalent Conjugation of Synthetic Peptides with Recombinant Proteins Mediated by Host-Guest Chemistry

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Materials and methods

The methyl viologen-modified cyclic and linear peptides were purchased from CPC Scientific Inc. (Sunnyvale, CA, USA) with purity > 95% and used without further purification. Cucurbit[8]uril (CB[8]), methyl viologen (MV) N,N'-diisopropylethylamine (DIPEA), dry dimethyl sulfoxide (DMSO) and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich. Peptide chemical structure and purity are shown in Fig. S1A.

Synthesis of FITC-modified peptide

For fluorescence polarisation experiments, the linear RGD-MV peptide was N-terminally labelled with fluorescein by incubating 10 mM peptide with 25 mM FITC in 98% v/v dry DMSO and 2% v/v DIPEA for 3 h at room temperature (RT). The reaction mixture was purified by reverse phase high performance liquid chromatography (RP-HPLC) on a C18 column. Chemical structure and purity is shown in Fig. S1B.

Plasmid construction

A Tn3 cassette[1] with N-terminal tryptophan and $(Gly_4\text{-}Ser)_4$ linker (W-$(G_4S)_4$-Tn3) was amplified from an existing phagemid plasmid construct by standard PCR methodology using the primers *ggaaacagctatgaccatgattacg* and *ctatgagaattctcactatgcggccccattcagatcc*. The cassette was cloned into an appropriately digested mammalian cell expression vector such that the W-$(G_4S)_4$-Tn3 construct was extended at the C-terminus by hexahistidine and c-myc tags (W-$(G_4S)_4$-Tn3-H_6-Myc).

Protein expression and purification

The Fc and Tn3 domains were expressed in Chinese hamster ovary (CHO) cells by standard methods using the following procedures. CHO-CEP6 cells (MedImmune) were maintained in CD-CHO medium (Life Technologies, Carlsbad, CA) containing 100 mg/l hygromycin B and 25 mg/L MSX. Aliquots of 30 mL CHO-CEP6 cells at 2.0 x 10^6 viable cells/mL were transferred into 125 mL flasks and placed in a 37 ºC shaking incubator set to 140 rpm with 5% CO_2 and 80% humidity. DNA was prepared by adding a total of 20 µg plasmid DNA to a 1.5 mL Eppendorf tube. The volume in each tube was made up to a total of 500 µL with 150 mM sodium chloride solution. The poly(ethyleneimine) (PEI) solution was prepared by adding a 200 µL volume of PEI Max working solution (1 mg/mL) to a 1.5 mL Eppendorf tube. The volume in each tube was made up to a total of 500 µL with 150 mM sodium chloride solution. The PEI solution was added to the DNA and vortexed for 10 s (DNA:PEI ratio 1:10). The PEI/DNA complex was incubated at room temperature for 1 min, and then added to the cells. Each flask was incubated in a 37 ºC shaking incubator set to 140 rpm with 5% CO_2 and 80% humidity. After a minimum of 4 h, a 9 mL volume of feed (30% initial volume) was added. The cultures were incubated for seven days in a 34 °C shaking incubator set to 140 rpm with 5% CO_2 and 80% humidity. Following incubation, the supernatant was harvested by transferring the content of the flasks to 50 mL Falcon tubes and centrifuging at 1500 rpm for 20 min followed by filtration through a 0.22 µm Steriflip filter.

Fc domains were purified by affinity chromatography on an ÄKTAxpress with MabSelectSuRe columns (GE Healthcare, Little Chalfont, UK). Columns were equilibrated in 1x Dulbecco's phosphate buffered saline (DPBS) (Life Technologies). Elution of the bound material was done with 0.1 M sodium citrate, pH 3, into collection blocks containing one fifth volume of 1 M Tris-HCl, pH 9. The samples were further purified by size exclusion chromatography on a HiLoad 16/600 Superdex 200 column (GE Healthcare) with isocratic elution at a flow rate of 1 mL/min 1x DPBS.

Tn3 domains were purified by affinity chromatography using the ÄKTAxpress with HisTrap Excel columns (GE Healthcare). The cleared expression supernatant was loaded onto the pre-equilibrated column (1x DPBS, 10 mM imidazole) and washed with 5 column volumes of 2x DPBS, 20 mM imidazole followed by elution of the bound material with 2x DPBS, 400 mM imidazole. The samples were further purified by size exclusion

chromatography on a HiLoad 16/600 Superdex 75 column (GE Healthcare) with isocratic elution at a flow rate of 1 mL/min 1x DPBS.

The concentration of the proteins was determined by absorption at 280 nm using theoretically calculated extinction coefficients. Protein purity assessed by polyacrylamide gel electrophoresis (PAGE) and size exclusion HPLC is shown in Figs 3A and 2B, respectively.

Isothermal calorimetry (ITC)

Isothermal titration experiments were performed on a Microcal Auto-iTC200 (Malvern Inc., Malvern, UK) in 10 mM sodium phosphate buffer, pH 7, at 25 °C (unless indicated otherwise), using 750 rpm stirring speed. Typically, an initial volume of 0.5 µL followed by consecutive volumes of 2 µL were injected for a total of 19 injections (36.5 µL total titrant volume), all spaced by 120 s. For precomplex formation, a 500 µM MV-modified peptide solution was titrated into 50 µM CB[8]. For ternary complex formation, 500 µM CB[8] was titrated in presence of 1 mM MV-modified peptide into a protein solution containing 50 µM N-terminal Trp/Tyr or Ser residues. Data was fitted to the one-set-of-sites model with Origin 7.0.

Tryptophan fluorescence spectroscopy

The fluorescence of the Trp residues of the protein was quantified using an F-7000 spectrophotometer with monochromator accessory (Hitachi High Technologies, Tokyo, JP) and an excitation wavelength of 295 nm. cRGD-MV and CB[8] were concentrated at 50 µM, the Fc domains at 25 µM (corresponding to 50 µM N-terminal Trp or Ser residues) in 10 mM sodium phosphate buffer, pH 7, 0.01% polysorbate 20.

Fluorescence polarization spectroscopy

The fluorescein-labelled FITC-RGD-MV was diluted to 300 nM in 10 mM phosphate buffer, pH 7, 0.01% polysorbate 20 and spiked with increasing amounts of CB[8] and Trp-Fc, respectively. The fluorescence polarization of the fluorescein residue was determined in a Wallac Victor2 plate reader (Perkin Elmer, Waltham, MA) with excitation and emission filters at 485 nm and 530 nm, respectively. Free fluorescein was used as a standard and set to a polarization value of $80 \cdot \times 10^{-3}$.

Multi-angle static laser light scattering (MALLS)

The change in molecular weight (M_w) upon complex formation was followed by MALLS. Samples (25 µM of all components in 10 mM phosphate buffer, pH 7) were filtered (0.45 µM PVDF) and directly infused into the light scattering detector (DAWN HELEOS II (Wyatt Technology, Santa Barbara, CA, USA) using an infusion syringe pump (PHD ultra, Harvard Apparatus, Holliston, MA) at a flow rate of 0.5 mL min^{-1}.

Asymmetric flow field flow fractionation (AF4)

The method of Moreth et al.[2] was adapted. Briefly, a HPLC system (Agilent, 1100 series) was used to control flow to an Eclipse DualTech (Wyatt Technology); the eluent used was 10 mM phosphate buffer, pH 7. Species were separated over a 1 kDa cut-off polyether sulfone (PES) membrane (Wyatt Technology, cat. no. 2725) with a 350 µm spacer. 20 µL samples of Trp-Tn3 (125 µM) with different molar ratios of cRGD-MV⊂CB[8]:Trp-Tn3 were injected. The detector flow was set at 1 mL/min and the cross flow was set at 2.0 mL/min from 0 to 15 min, 2.0 to 0.2 mL/min from 15 to 20 min, and 0.2 mL/min from 20 min to 30 min. Elution of protein species was followed by absorbance at 280 nm.

Stopped-flow kinetics measurements

The assembly kinetics of the ternary complex were followed by stopped-flow fluorescence spectroscopy (SX20, Applied Photophysics, Leatherhead, UK) with excitation at 280 nm and emission measured above 320 nm. A solution of 0.5 µM Trp-Fc (corresponding to 1 µM N-terminal Trp residues) in 10 mM sodium phosphate buffer, pH 7, was injected together with solutions of the cRGD-MV⊂CB[8] precomplex (5, 10 and 15 µM) in a 1:1 volume ratio into the reaction cell. Experiments were performed at temperatures including 7.5, 10 and 15 °C. Data was collected for 8 repeats of each condition and analysed according to Appel *et al.*[3]

Briefly, data for each repeat and condition was fit to a single-exponential decay function using Graphpad Prism 6.0 yielding the observed rate constants (k_{obs}). Data of the initial 2 ms of each experiment was excluded from the fitting in order to avoid mixing artifacts. Values for k_{obs} at different temperatures were then plotted against the respective concentrations of the cRGD-MV⊂CB[8] precomplex yielding the association and dissociation rate constants (k_a and k_d, respectively) for the complexation of Trp-Fc with cRGD-MV⊂CB[8]. According to the Arrhenius law, activation energies of the association (Ea_a) and dissociation (Ea_d) could then be obtained by

plotting 1/T versus ln(k_a) and ln(k_d), respectively. Furthermore, this allowed for the prediction of values for k_a and k_d at elevated temperatures such as 25 °C and 37 °C (assuming linearity of the Arrhenius plot in this temperature range).

Surface plasmon resonance (SPR) experiments

SPR analysis was conducted on a Biacore T100 instrument (GE Healthcare) with a Series S CM5 chip (GE Healthcare). Immobilization was performed using the Amine coupling kit (GE Healthcare). Integrin $\alpha_v\beta_3$ (Millipore, cat. no. CC1021, 20 µg/mL) in HBS-P buffer (GE Healthcare) supplemented with 1 mM $MgCl_2$ was immobilized for 600 s using a flow rate of 10µl/min resulting in an immobilization level of 85 RU.
For binding experiments, Trp-Fc (25 µM N-terminal Trp residues) with 25 µM peptide and CB[8] in running buffer (10 mM sodium phosphate buffer, pH 7, supplemented with 1 mM $MgCl_2$ and 0.02 % polysorbate 20) was injected at 30 µL/min with a contact time of 200 s followed by dissociation for 600 s. For competition experiments, linear GRGDSP peptide (Sigma-Aldrich, St. Louis, MO, USA) was spiked into the injection sample.

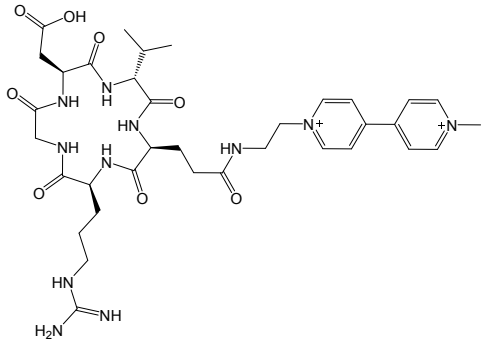
References

[1] R. N. Gilbreth, B. M. Chacko, L. Grinberg, J. S. Swers, M. Baca, *Protein Eng. Des. Sel.* **2014**, *27*, 411-418.

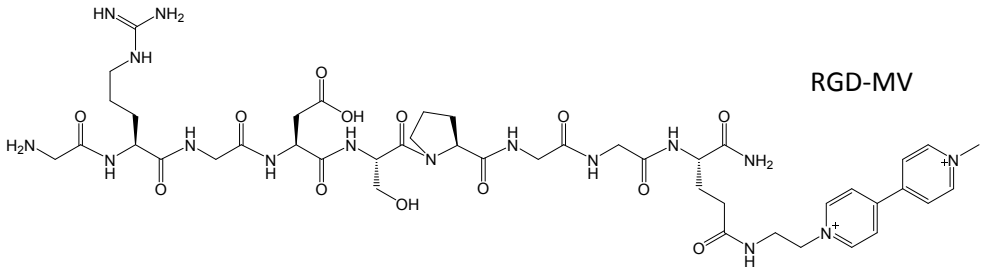
[2] J. Moreth, K. S. Kroker, D. Schwanzar, C. Schnack, C. A. F. von Arnim, B. Hengerer, H. Rosenbrock, L. Kussmaul, *Biochemistry* **2013**, *52*, 1466-1476.

[3] E. A. Appel, R. A. Forster, A. Koutsioubas, C. Toprakcioglu, O. A. Scherman, *Angew. Chem. Int. Ed.* **2014**, *53*, 10038-10043, *Angew. Chem.* **2014**, *126*, 10202–10207.

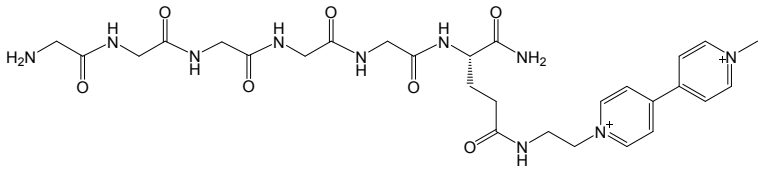
Supplementary figures and tables



cRGD-MV



RGD-MV

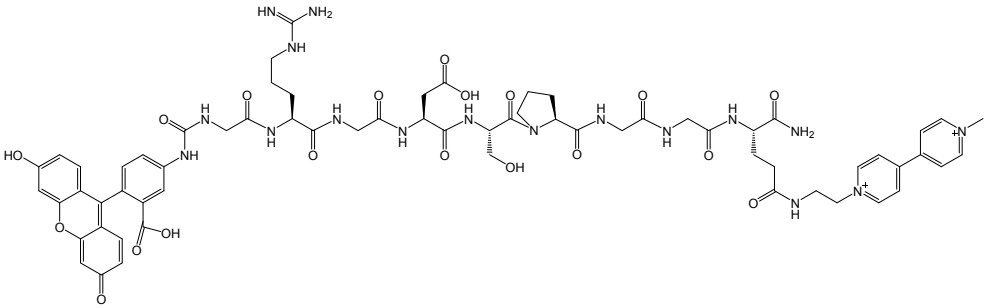


G_5-MV

Peptide	RP-HPLC % purity	MW (theor) g/mol	MW (exp) g/mol
cRGD-MV	97.2	752.9	752.6
RGD-MV	97.3	1025.8	1024.5
G_5-MV	95.7	628.4	627.3

Fig. S1A Chemical structure of MV modified peptides used in this study. Cyclic RGDvE (cRGD-MV), linear GRGDSPGGE (RGD-MV) and linear G_5E (G_5-MV) were purchased as aminoalkyl viologen (MV) at the Glu residue. The purity and molecular weight data obtained from the manufacturer (CPC Scientific Inc.) are reported in the table.

i)



FITC-RGD-MV

ii)

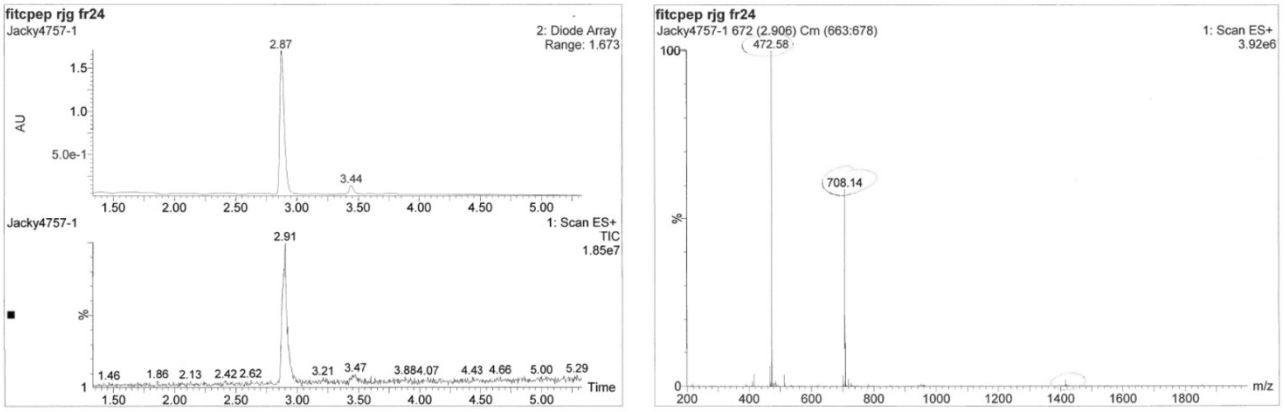


Fig. S1B i), Chemical structure of the fluorescein modified RGD-MV peptide (Fig. S1A). **ii)**, Liquid chromatography–mass spectrometry (LC-MS) traces of the purified FITC-RGD-MV peptide; the diode array recorded the absorbance at 220 nm.

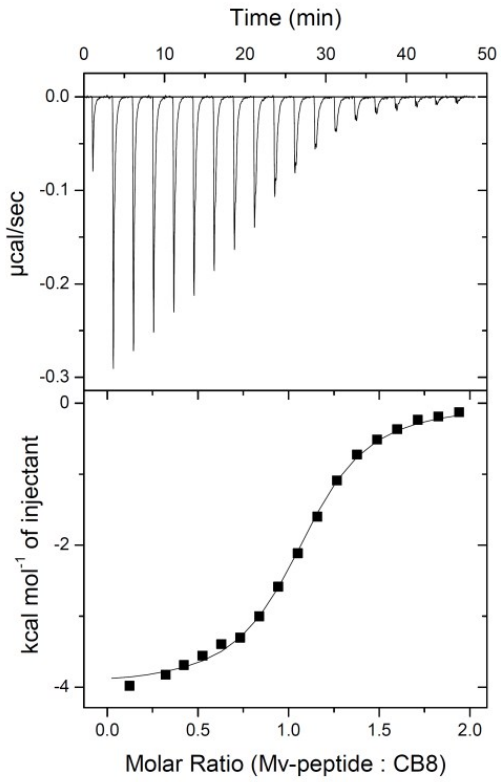


Fig. S2 Characterisation of the precomplex assembly by ITC. Over 19 injections (2 µL each, following an initial 0.5 µL inject), a 500 µM solution of cRGD-MV was titrated into a solution of 50 µM CB[8]. The cRGD-MV⊂CB[8] precomplex was formed with a 1:1 stoichiometry and an equilibrium binding constant K_a of 6.3 x 10^5 M^{-1}.

Table S1 Thermodynamic data for the interaction of cRGD-MV⊂CB[8] with the Fc domains derived by isothermal calorimetry at 25 °C (see also Fig. S4).

Guest	K_a [M^{-1}]	ΔH [kcal mol^{-1}]	TΔS [kcal mol^{-1}]
Trp-Fc	2.3 (± 0.2) x 10^5	-17.9 ± 0.4	-10.6 ± 0.6
Tyr-Fc	4.2 (± 0.2) x 10^4	-21.3 ± 1	-14.9 ± 1.2
Ser-Fc[a]	--[a]	--[a]	--[a]

[a] No interaction observed by ITC with tested host-guest concentrations.

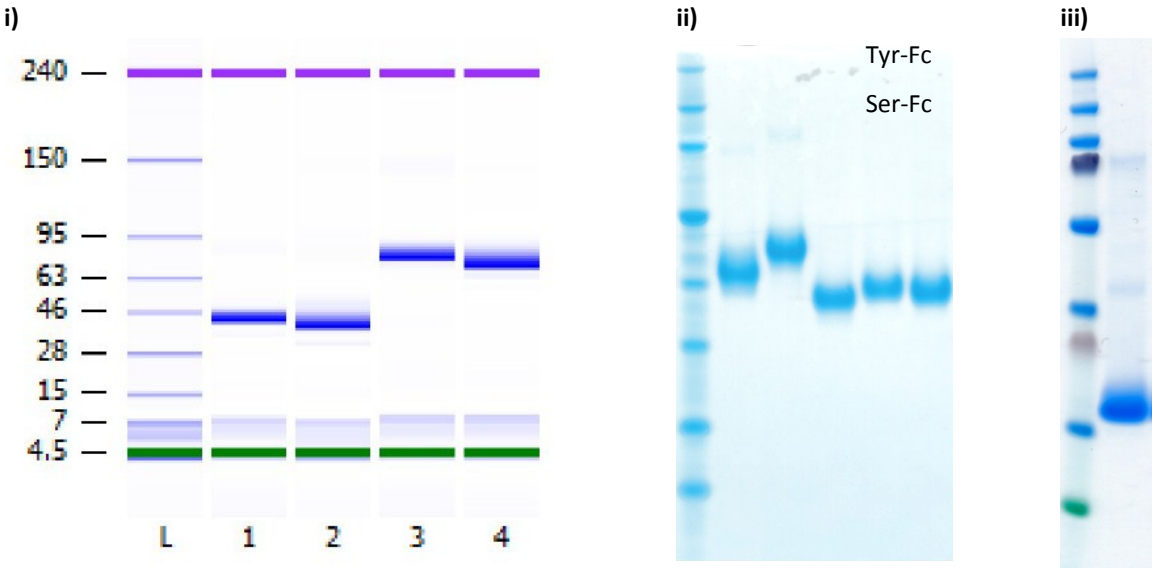


Fig. S3 A, Gel electrophoresis data of **i)** Trp-Fc in lane 2 (reducing) and lane 4 (non-reducing); **ii)** Tyr-Fc and Ser-Fc and iii) Trp-Tn3. Trp-Fc was analysed by 2100 Bioanalyzer (Agilent), the other Fc domains and Trp-Tn3 were analysed by standard SDS-PAGE using premade gels NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels (ThermoFisher Scientific) and PageRuler™ Plus Prestained Protein Ladder 10 to 250 kDa (ThermoFisher scientific). Other protein lanes are shown in i) and ii) but are not reported here since they are not related to this work.

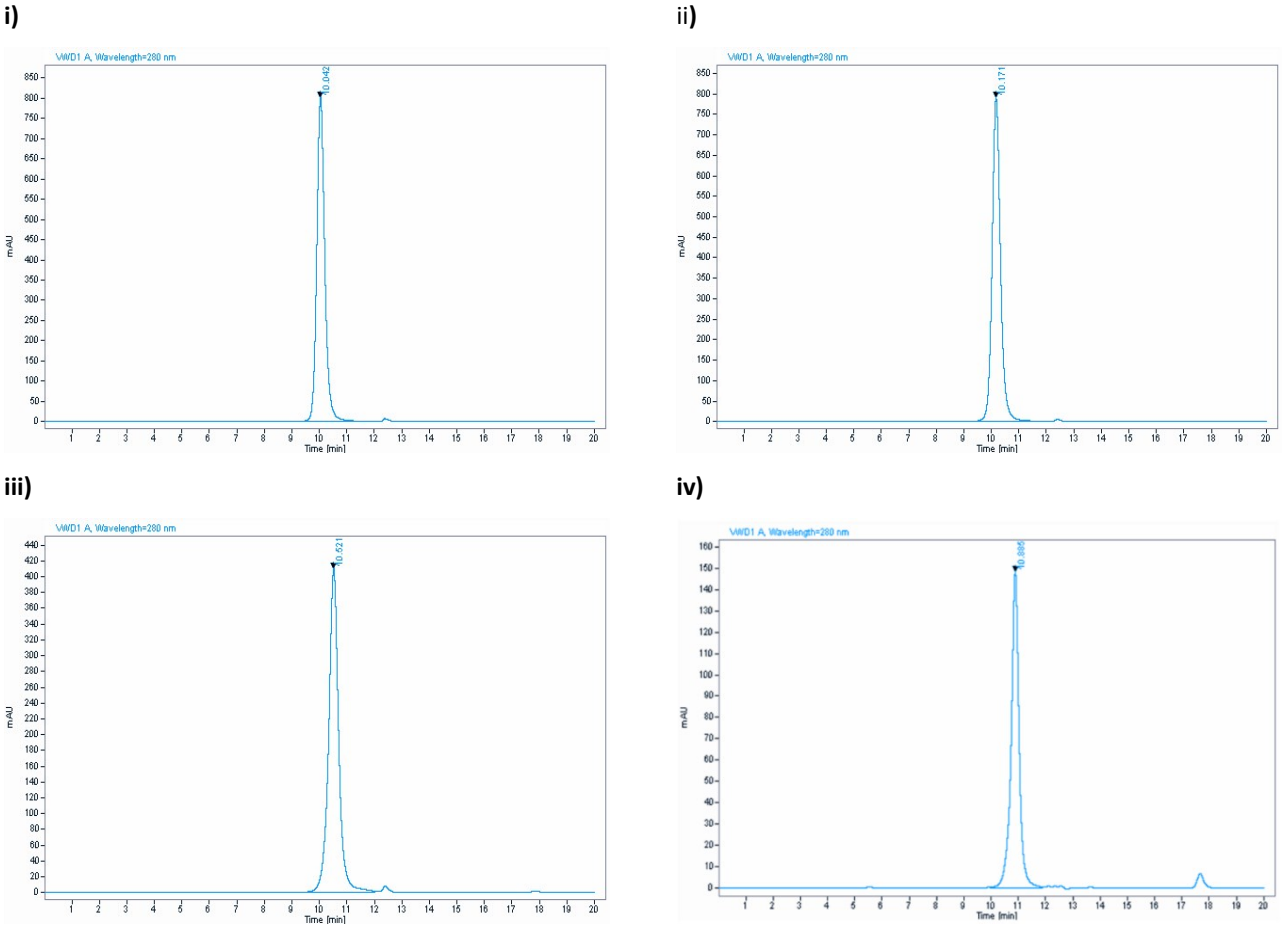


Fig. S3 B, HP-SEC traces of the purified Fc and TN3 domains used: **i)** Trp-Fc; **ii)** Tyr-Fc; **iii)** Ser-Fc and **iv)** Trp-Tn3, respectively. All of the traces were followed at 280 nm.

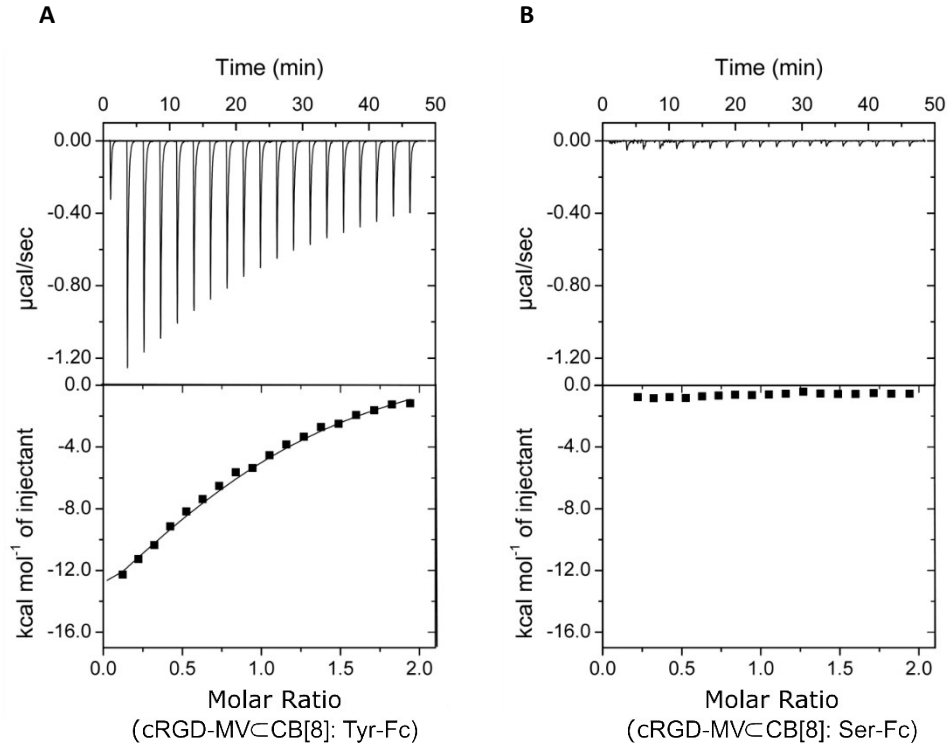


Fig. S4 Characterisation by isothermal titration calorimetry (ITC) of the binding of the cRGD-MV⊂CB[8] precomplex to Fc domains harbouring N-terminal tyrosines (Tyr-Fc, **A**) or serines (Ser-Fc, **B**). Over 19 injections (2 µL each, following an initial 0.5 µL inject), a 500 µM solution of cRGD-MV⊂CB[8] was titrated into a solution of the corresponding Fc-domain (50 µM N-terminal residues).

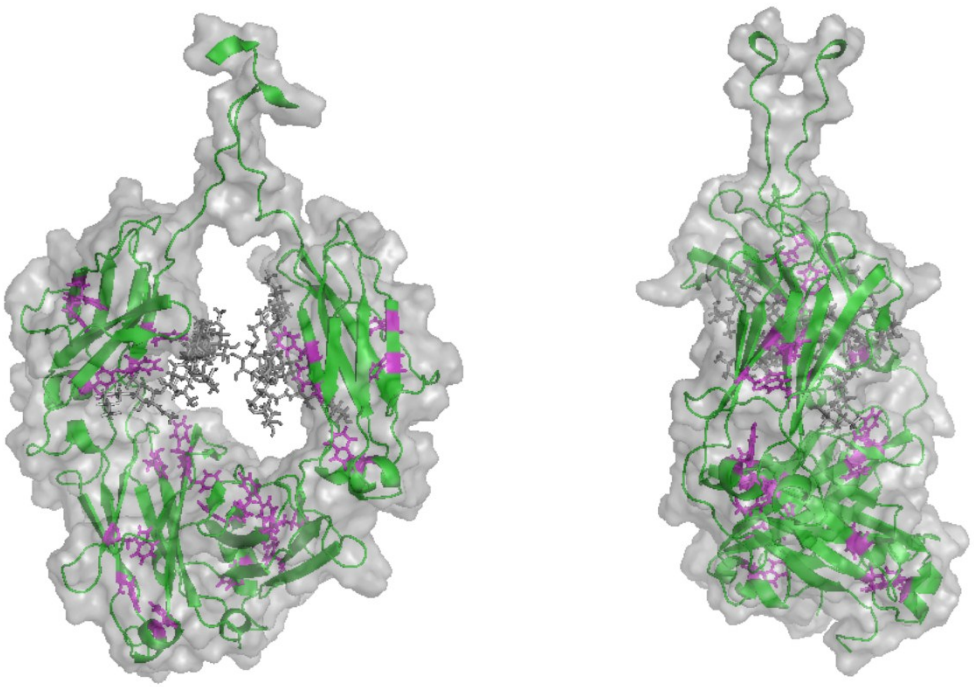


Fig. S5 Crystal structure of the Fc domain shown as ribbon (green) and space-filling models (light grey), with the carbohydrate (dark grey stick model). The hydrophobic residues (Trp and Phe) are represented as purple sticks to highlight that they are part of the hydrophobic core.

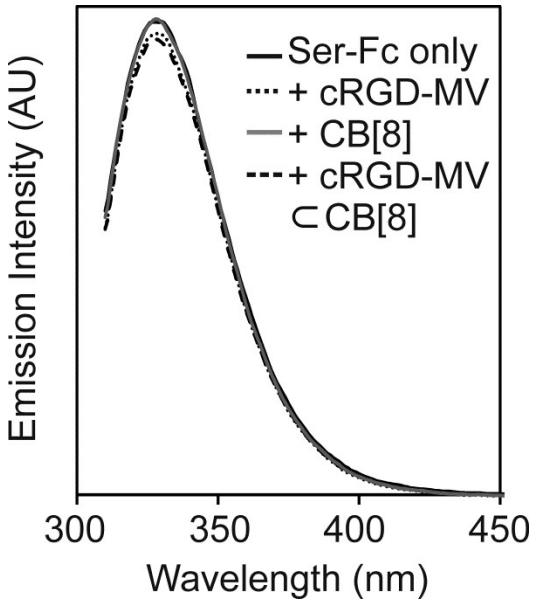


Fig. S6 Changes in tryptophan fluorescence of Ser-Fc (50 µM N-terminal Ser) were followed by the addition of equimolar amounts of cRGD-MV and CB[8] alone or in precomplex (cRGD-MV⊂CB[8]).

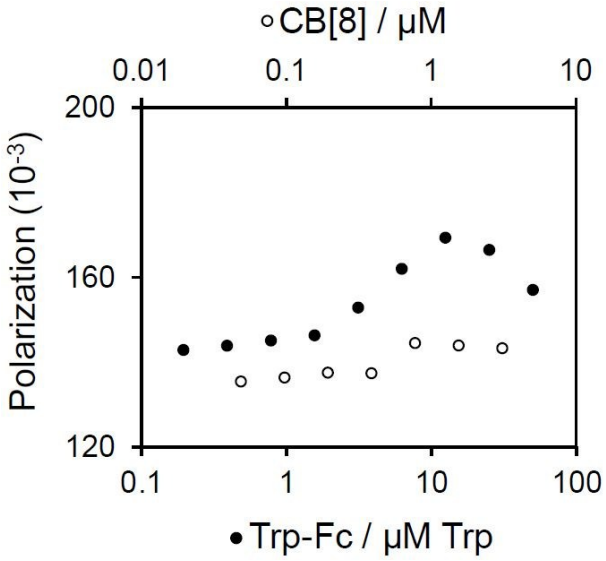


Fig. S7 Change in molecular size during complex assembly. Fluorescence polarisation of a fluorescently labelled peptide (FITC-RGD-MV, 0.3 µM) was measured in presence of increasing concentrations of CB[8] (clear dots) and in precomplex with CB[8] (10 µM) with increasing concentrations of Trp-Fc (black dots; Trp-Fc concentration represents N-terminal Trp residues).

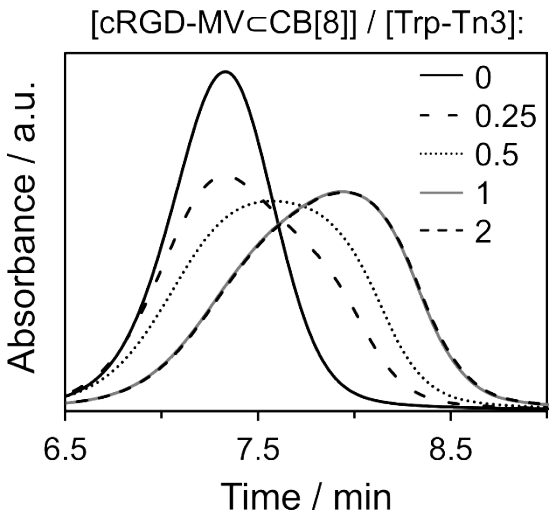


Fig. S8 Molecular size of the peptide-protein conjugations. The change in molecular size upon peptide-protein conjugation is demonstrated by AF4 elution curves of Trp-Tn3 in presence of increasing molar ratios of cRGD-MV⊂CB[8]:Trp-Tn3. Data at ratio 2 are shown as dashed to allow for the visualisation of data at ratio 1.

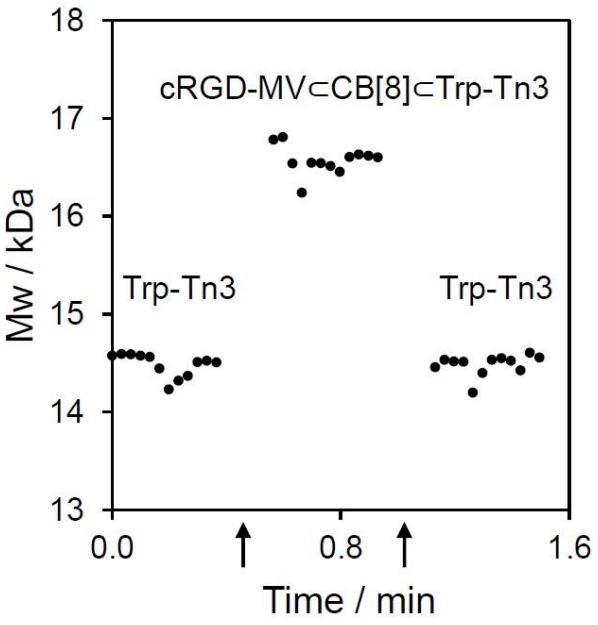


Fig. S9 Change in molecular size of Trp-Tn3 (25 µM) upon complex formation with cRGD-MV⊂CB[8]. The molecular weight (M_w) of Trp-Tn3 alone or in complex (cRGD-MV⊂CB[8]⊂Trp-Tn3) was determined by multi-angle static laser light scattering (MALLS). Arrows indicate the solution changes during direct infusion into the detector.

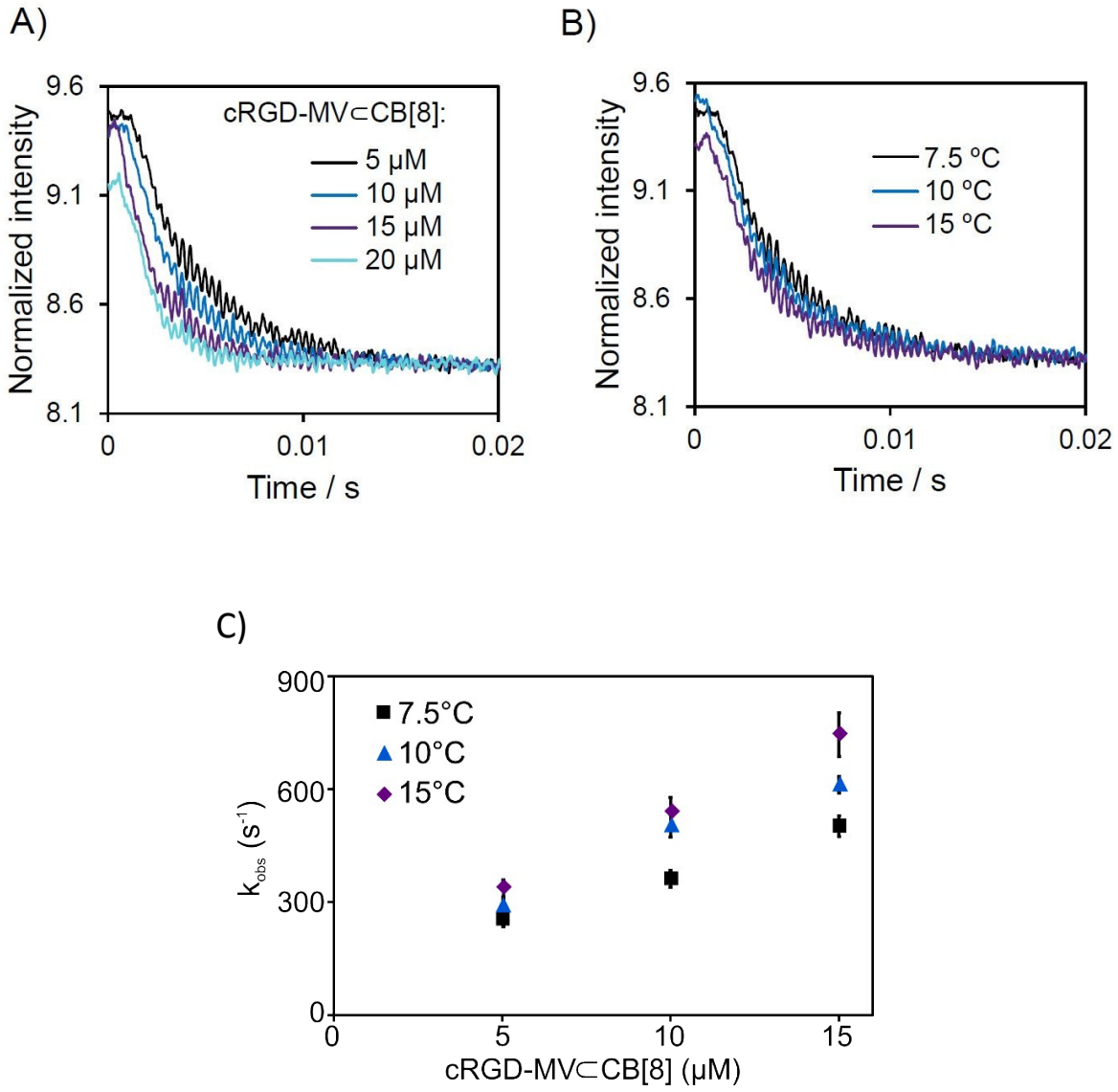


Fig. S10 Assembly kinetics of the ternary complex (cRGD-MV⊂CB[8])$_2$⊂Trp-Fc. **A)** Assembly was followed by the decrease in fluorescence of the 2nd guest Trp (excitation wavelength 280 nm). Concentration-dependent kinetics were obtained by mixing Trp-Fc (1 µM N-terminal Trp residues) with increasing concentrations of cRGD-MV⊂CB[8] from 5 – 20 µM (A, data shown for T = 7.5 °C). **B)** Temperature was varied from 7.5 – 15 °C (B, data shown for [cRGD-MV⊂CB[8]] = 5 µM). **C)** Fitting of the fluorescence traces to a single-exponential decay function yielded the observed rate constants (k_{obs}). Plotting of k_{obs} in dependence of the cRGD-MV⊂CB[8] concentration at different temperatures led to the association and dissociation rate constants (k_a and k_d, respectively) for the complexation of Trp-Fc with cRGD-MV⊂CB[8]. According to the Arrhenius law, activation energies of the association (Ea_a) and dissociation (Ea_d) could then be obtained by plotting 1/T versus ln(k_a) and ln(k_d), respectively.