Supporting Information

High-affinity RGD-knottin peptide as a new tool for rapid evaluation of the binding strength of unlabeled RGD-peptides to $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrin receptors

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General

UPLC analysis was performed on a Waters Acquity Ultra Performance LC System, equipped with a Waters Acquity UPLC BEH130 C18 1.7 µm column. A linear gradient of 5-55% ACN (0.05% TFA) in H_2O (0.05% TFA) was used. Peptides were purified by preparative HPLC (Waters Prep LC) on an RP-C18 column (Reprosil-Pur 120 C18-AQ 150x20 mm, Dr. Maisch GmbH, Ammerbuch, Germany) using an ACN/H_2O gradient (5-65%) including 0.05% TFA.

Synthesis protocol for biotinylated peptides 1a-c

Biotinylated peptides **1a-c** were synthesized in two steps. First, the linear peptide was synthesized via Fmoc-based solid-phase peptide synthesis (SPPS) on a Rink-amide resin using standard protocols. Second, linear peptide was folded using following protocol (example): Linear precursor of **1a** (20 mg, 5.2 µmol) was dissolved at 0.1 µM in miliQ water containing 2.2 M guanidinium hydrochloride (GuHCl), further referred to as peptide solution. Folding buffer (twice the volume of peptide solution) was made by dissolving cysteine (55.8 mg, 460 µmol) and cystine (13.4 mg, 55.7 µmol) in 1 M GuHCl and 25 mM Tris buffer (pH 8). After sonication and filtration, peptide solution was slowly added and reacted 24 h at r.t. without stirring. The reaction was quenched with 10% TFA solution until pH < 4 and the oxidized product subsequently purified via HPLC. $[M+3H]^{3+}$ calculated for **1a**: 1269.18, found: 1270.26 (Figure S1).

Synthesis protocol for peptides 2a-c

Peptides **2a-c** were synthesized via Fmoc-based solid-phase peptide synthesis (SPPS) on a 2-chlorotrityl resin using standard protocols, starting with coupling of Gly to prevent racemization in backbone cyclization. For **2a+b**, a lysine residue that carried an Fmoc-protected side chain and an ivDde-protected *N*-terminus was used. After Fmoc-deprotection, subsequent linker coupling, and biotinylation of the lysine side chain, the ivDde group was removed using 2% hydrazine in DMF, and the remaining amino acids were coupled. Then, the peptide was cleaved with 2% TFA/DCM and isolated as a fully side-chain protected peptide, which was then backbone-cyclized using the following protocol: to a solution of fully side-chain protected peptide in DCM (0.3 mM) was added PyBop (2 eq) and after 20 min *N*,*N*-diisopropylethylamine (10 eq). After reaction overnight at room temperature, solvent was removed in vacuum and 1.5 mL per 40 µmol peptide of 95% TFA were added to fully deprotect the cyclized peptide. After 2h, peptide was precipitated 2 times with 1:1 Ether/*N*-pentane, and after lyophilization, the peptide was purified via HPLC. *m/z* calculated for **2a**: 1119.54, found: 1119.48 (Figure S2).

Spectra

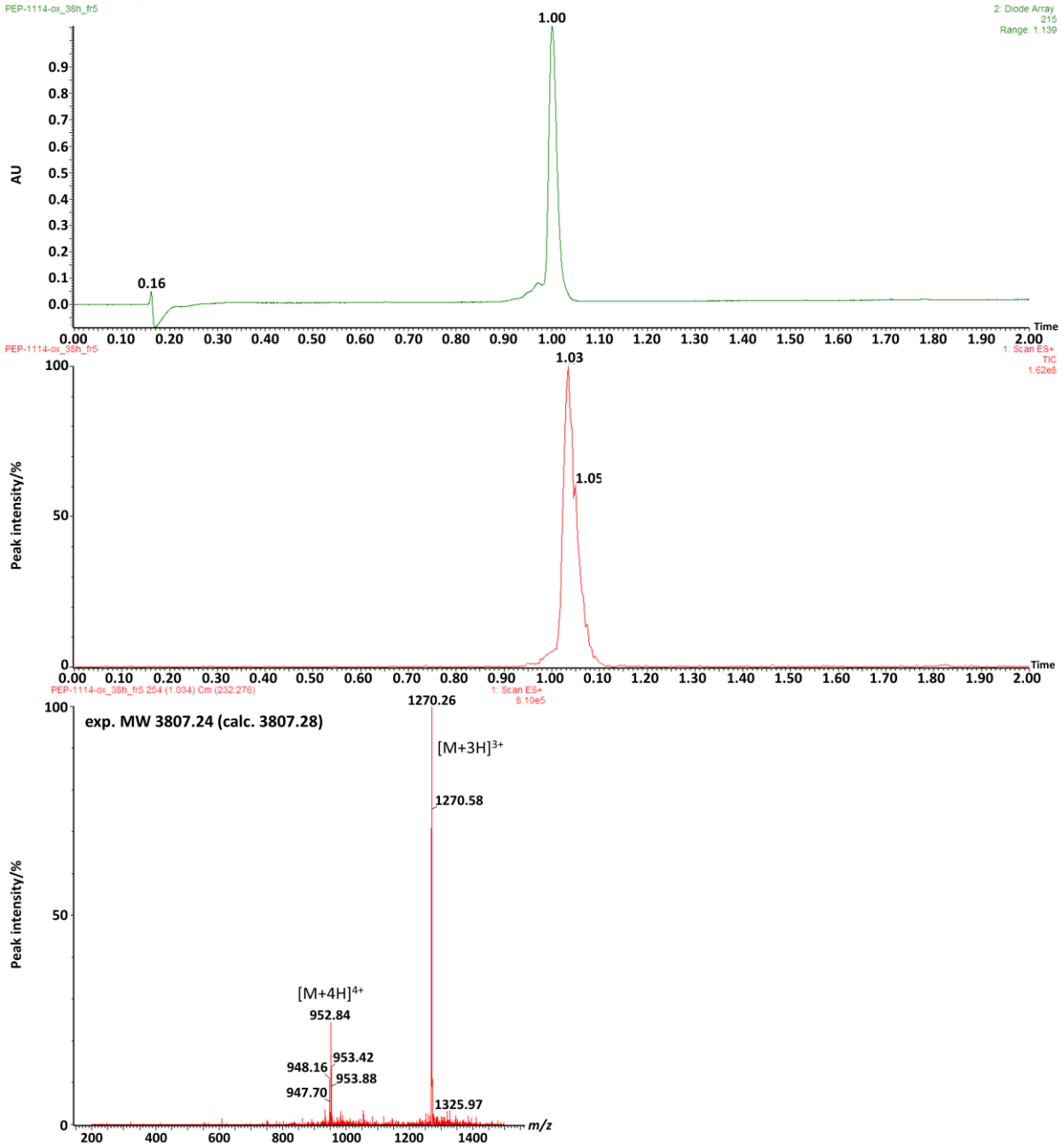

Figure S-1. UPLC/MS spectrum of purified knottin-RGD peptide **1a** (top: UV detection at 215 nm, middle/bottom: ESI-MS detection).

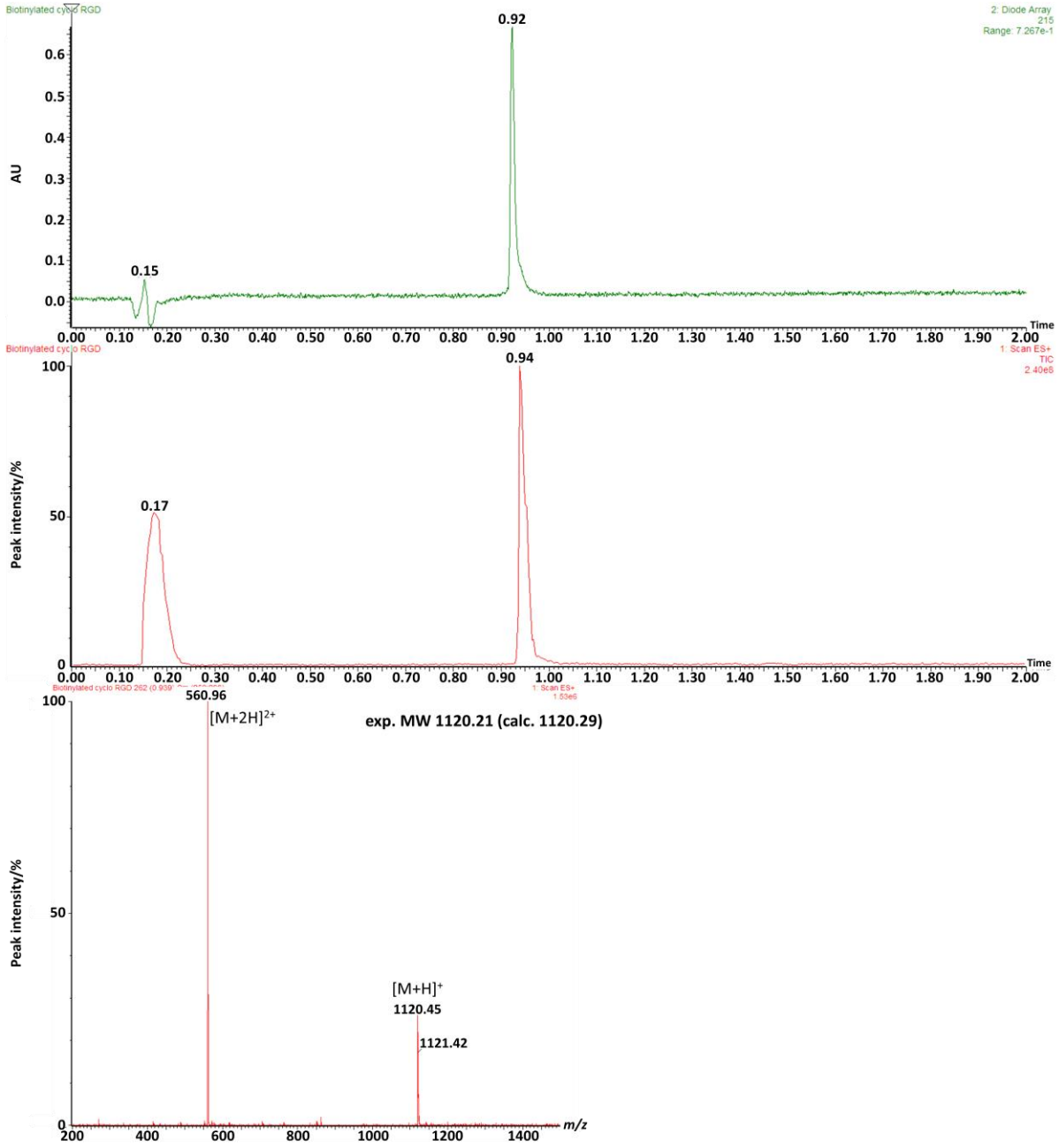

Figure S-2. UPLC/MS spectrum of purified cyclic-RGD peptide **2a** (top: UV detection at 215 nm, middle/bottom: ESI-MS detection).

ELISA

All integrins were dissolved according to manufacturer's protocol and stored in 25 µL aliquots of 100 µg/mL in PBS. For coating of one 96 well plate, 2 aliquots were thawed and dissolved in 9.95 mL of cold coating buffer for a 0.5 µg/mL solution. After coating, the plates were subsequently sealed and stored at 4 °C overnight. Integrin was removed and plates were blocked with 150 µL 1% I-Block solution for 1h at room temperature. The following washing and incubation steps were performed according to manuscript. ABTS substrate buffer was prepared by mixing substrate buffer pH 4, 20 g/L ABTS and 3% H_2O_2 in the volumetric ratio 10/0.25/0.02. A schematic setup of the binding ELISA and competition ELISA is shown in Figure S3. Exemplary ELISA experiment results are shown in Figures S4 (binding ELISA) and S5 (competition ELISA).

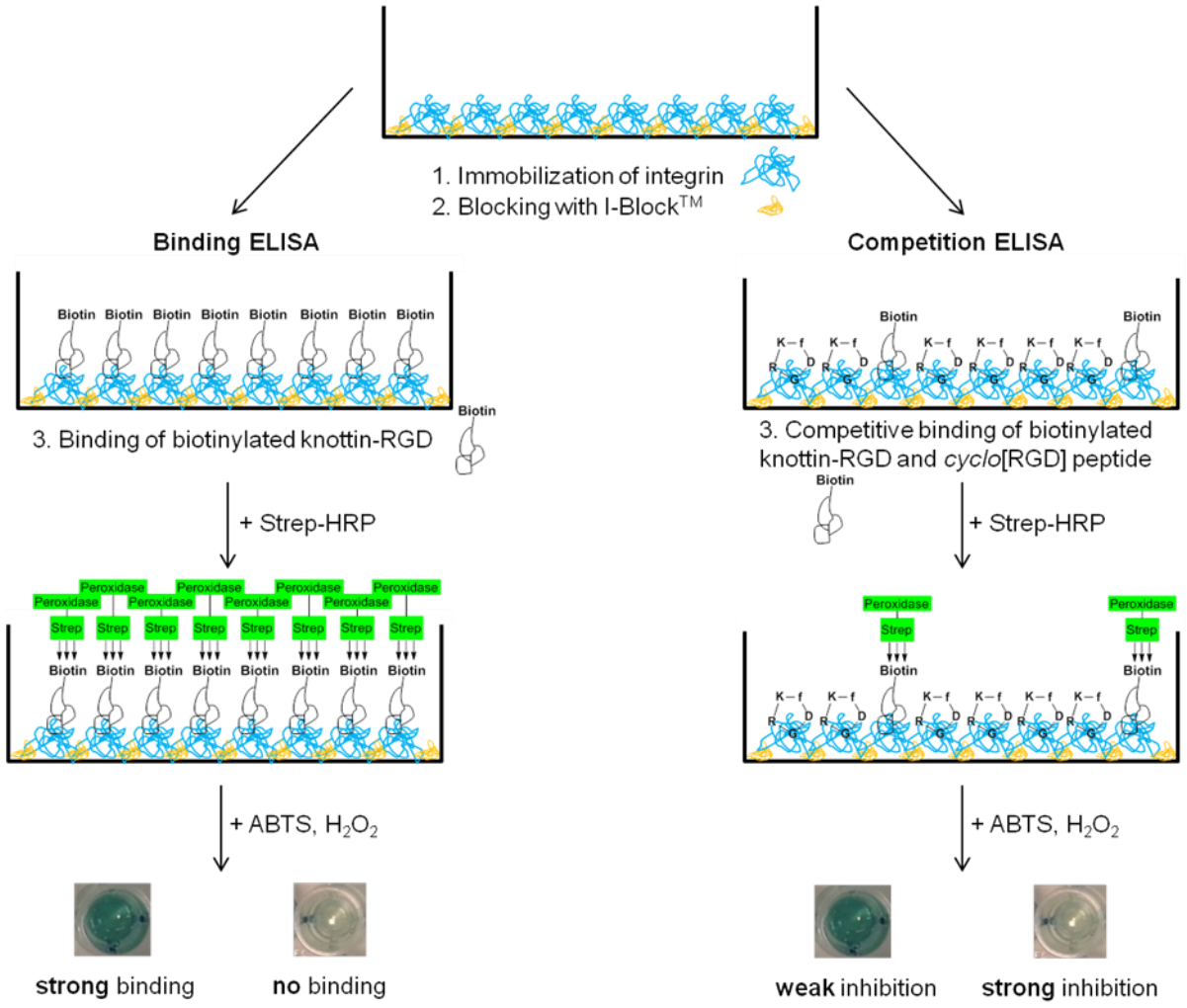

Fig. S-3. Schematic setup of the direct binding ELISA (left) and competition ELISA (right). Color change is induced by biotin-bound streptavidin-horseradish peroxidase ("Peroxidase-Strep").

Plate A			$c_{comp.\ Peptide}$ [µM]	10			1			0.1			Peptide 2b [µM] A: 10, B: 1, C: 0.1, D: 0		
Integrin	**Washing**	**Peptide**	**Polysorp**	1	2	3	4	5	6	7	8	9	10	11	12
$\alpha_5\beta_1$ (0.5 µg/mL)	**with** Ca,Mg	**1a**	A	1.39	1.29	1.12	0.77	0.93	0.92	0.44	0.47	0.44	0.19	0.18	0.18
		1b	B	0.33	0.27	0.28	0.20	0.20	0.21	0.16	0.19	0.20	0.18	0.15	0.17
		1c	C	0.29	0.24	0.24	0.18	0.19	0.19	0.20	0.19	0.19	0.19	0.16	0.18
		2a	D	0.38	0.35	0.30	0.27	0.25	0.26	0.19	0.20	0.18	0.18	0.17	0.17
	without Ca,Mg	**1a**	E	0.73	0.68	0.62	0.40	0.42	0.39	0.22	0.25	0.25	0.14	0.17	0.16
		1b	F	0.25	0.22	0.29	0.18	0.18	0.17	0.18	0.16	0.15	0.15	0.16	0.16
		1c	G	0.25	0.22	0.24	0.13	0.15	0.17	0.18	0.19	0.16	0.16	0.13	0.14
		2a	H	0.30	0.31	0.29	0.22	0.24	0.23	0.17	0.16	0.15	0.14	0.16	0.14

Figure S-4. Absorbances determined in a binding ELISA experiment. Background colors range from white (O.D. 0) to green (O.D. 4). The data show that only knottin-RGD peptide **1a** clearly binds to integrin $\alpha_5\beta_1$.

PLATE A		$c_{comp.\ Peptide}$ [µM]	30	10	3.3333	1.1111	0.3704	0.1235	0.0412	0.0137	0.0046	0.0015	0.0005	0.0002
Integrin	**comp. Peptide**	**Polysorp**	1	2	3	4	5	6	7	8	9	10	11	12
$\alpha_v\beta_3$ (0.5 µg/mL)	**1d**	A	0.18	0.19	0.18	0.23	0.73	1.48	1.70	1.82	1.74	1.68	1.73	1.85
		B	0.18	0.17	0.17	0.21	0.63	1.31	1.58	1.53	1.56	1.58	1.63	1.58
		C	0.21	0.14	0.17	0.21	0.59	1.23	1.49	1.54	1.47	1.64	1.64	1.69
	2c	D	0.20	0.19	0.25	0.40	0.63	0.99	1.21	1.30	1.43	1.62	1.58	1.66
		E	0.18	0.20	0.25	0.40	0.65	0.97	1.27	1.33	1.36	1.59	1.64	1.75
		F	0.18	0.20	0.27	0.39	0.73	1.10	1.36	1.44	1.51	1.58	1.62	1.82
	no peptide	G	1.83	1.78	1.69									
PLATE B		$c_{comp.\ Peptide}$ [µM]	30	10	3.3333	1.1111	0.3704	0.1235	0.0412	0.0137	0.0046	0.0015	0.0005	0.0002
Integrin	**Peptide**	**Polysorp**	1	2	3	4	5	6	7	8	9	10	11	12
$\alpha_v\beta_3$ (0.5 µg/mL)	**GRGDS**	A	0.42	0.72	1.22	1.46	1.69	1.72	1.75	1.72	1.63	1.75	1.72	2.45
		B	0.41	0.65	1.06	1.29	1.45	1.50	1.55	1.52	1.52	1.62	2.69*	2.24
		C	0.45	0.76	0.98	1.27	1.44	1.54	1.57	1.55	1.56	1.52	3.17*	2.15
	cylcoFYFDLRK	D	1.81	1.69	1.67	1.61	1.56	1.54	1.58	1.53	1.42	1.48	1.67	1.93
		E	1.79	1.64	1.73	3.28*	1.57	1.59	1.60	1.62	1.70	1.71	1.59	1.76
		F	1.84	1.67	1.71	1.66	1.64	1.66	1.67	1.58	1.70	1.72	1.63	1.73

Figure S-5. Absorbances determined in a competition ELISA experiment with integrin $\alpha_v\beta_3$. Background colors range from white (O.D. 0) to green (O.D. 4) and depend on the peptide and its concentration.* Those values were not considered for IC_{50} calculation.