Supplementary Material

Facile double-functionalization of Designed Ankyrin Repeat Proteins using click and thiol chemistries

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Supplementary Tables

Table S1. (A) Amino acid analysis of Ec1 expressed in Aha-containing medium and (B) conventionally expressed Ec1.

A

	pmol	residues (known)	pmol/ residue	pmol/residue within 15% error range	residues (calc.)	error (%)
Histidine	51.46	14	3.68	3.68	13.3	5.10
Serine	5.98	2	2.99		1.5	22.82
Arginine	14.59	4	3.65	3.65	3.8	5.81
Glycine	66.96	15	4.46	4.46	17.3	15.26
Aspartic acid	102.65	27	3.80	3.80	26.5	1.83
Glutamic acid	55.84	14	3.99	3.99	14.4	2.98
Threonine	28.21	7	4.03	4.03	7.3	4.06
Alanine	89.73	22	4.08	4.08	23.2	5.31
Proline	19.28	4	4.82		5.0	24.47
Lysine	36.01	10	3.60	3.60	9.3	7.01
Tyrosine	15.49	3	5.16		4.0	33.34
Methionine	**0.27**	**0**			**0.1**	
Valine	44.19	12	3.68	3.68	11.4	4.91
Isoleucine	28.69	8	3.59	3.59	7.4	7.39
Leucine	88.99	22	4.05	4.05	23.0	4.45
Phenylalanine	14.92	3	4.97		3.9	28.41
		average	4.04	3.87		11.54

B

	pmol	residues (known)	pmol/ residues	pmol/residue within 15% error range	residues (calc.)	error (%)
Histidine	68.68	14	4.91	4.91	13.5	3.69
Serine	2.00	2	4.88	4.88	1.9	4.20
Arginine	20.95	4	5.24	5.24	4.1	2.82
Glycine	80.88	15	5.39	5.39	15.9	5.87
Aspartic acid	141.03	27	5.22	5.22	27.7	2.55
Glutamic acid	3.69	14	3.99	3.99	14.4	2.98
Threonine	31.44	7	4.49	4.49	6.2	11.82
Alanine	121.80	22	5.54	5.54	23.9	8.70
Proline	22.97	4	5.67	5.67	4.5	11.28
Lysine	55.93	10	5.59	5.59	11.0	9.81
Tyrosine	15.91	3	5.30	5.3	3.1	4.11
Methionine	**3.10**	**1**	**3.10**		**0.60**	**39.15**
Valine	51.50	12	4.29	4.29	10.1	15.74
Isoleucine	34.31	8	4.29	4.29	6.7	15.79
Leucine	112.40	22	5.11	5.11	22.1	0.31
Phenylalanine	15.55	3	5.18	5.18	3.1	1.79
		average	4.97	5.09		8.85

Supplementary Figures

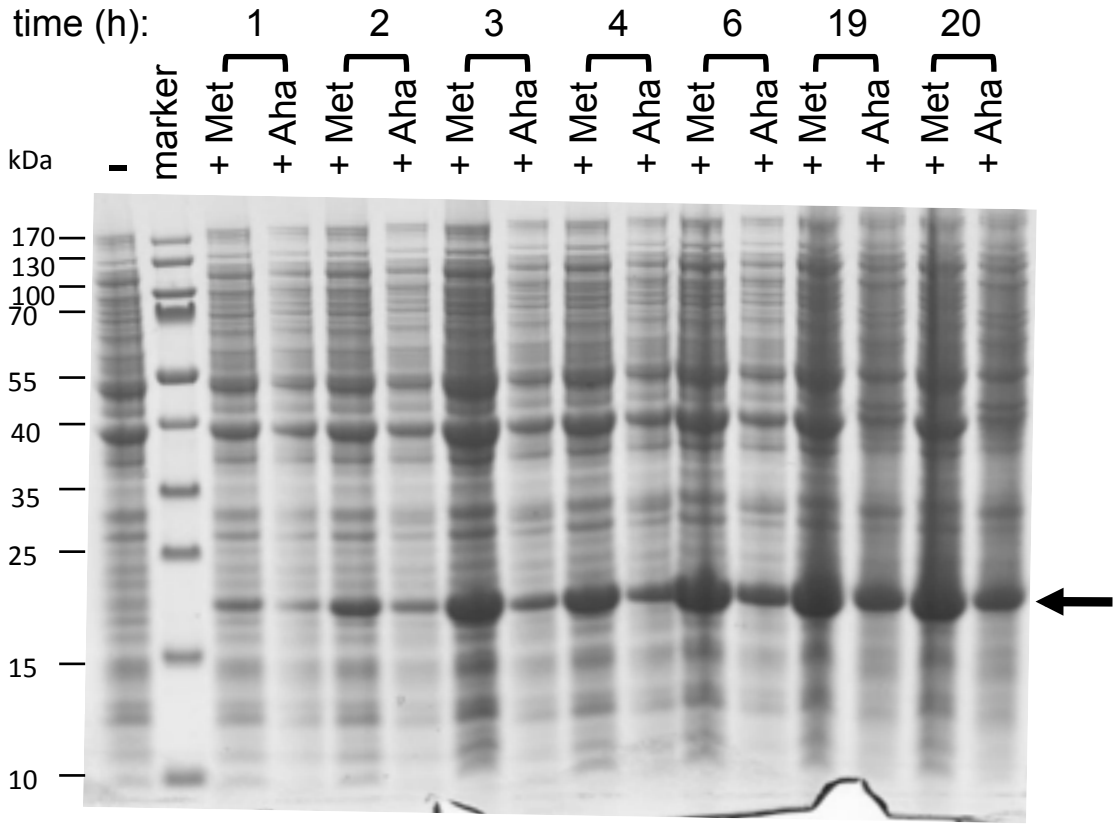

Figure S1. Induction of Ec1 as a function of time: *E. coli* B834 (DE3) was transformed with the plasmid pQIq-Ec1 and the DARPin expressed in 2 × YT medium (denoted as + Met) or M9 minimal medium supplemented with azidohomoalanine (denoted as + Aha) followed by analysis by 15% SDS PAGE. The arrow indicates the expected molecular size of Ec1. An aliquot of a non-induced culture was used as negative control (-).

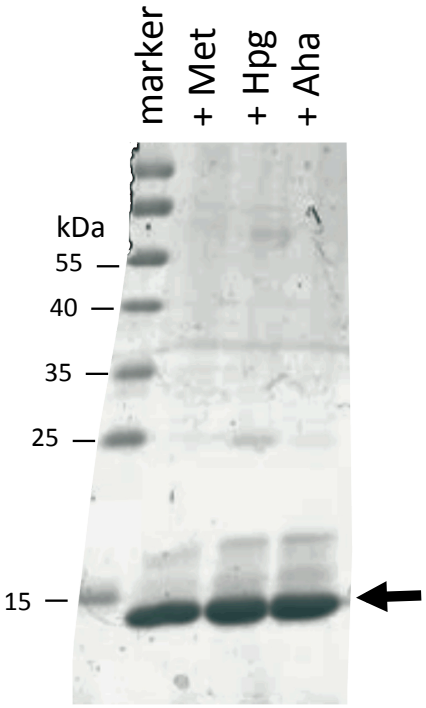

Figure S2. SDS PAGE analysis of IMAC-purified Ec1 expressed in M9 minimal medium supplemented with methionine (+ Met), homopropargylglycine (+ Hpg) or azidohomoalanine (+ Aha).

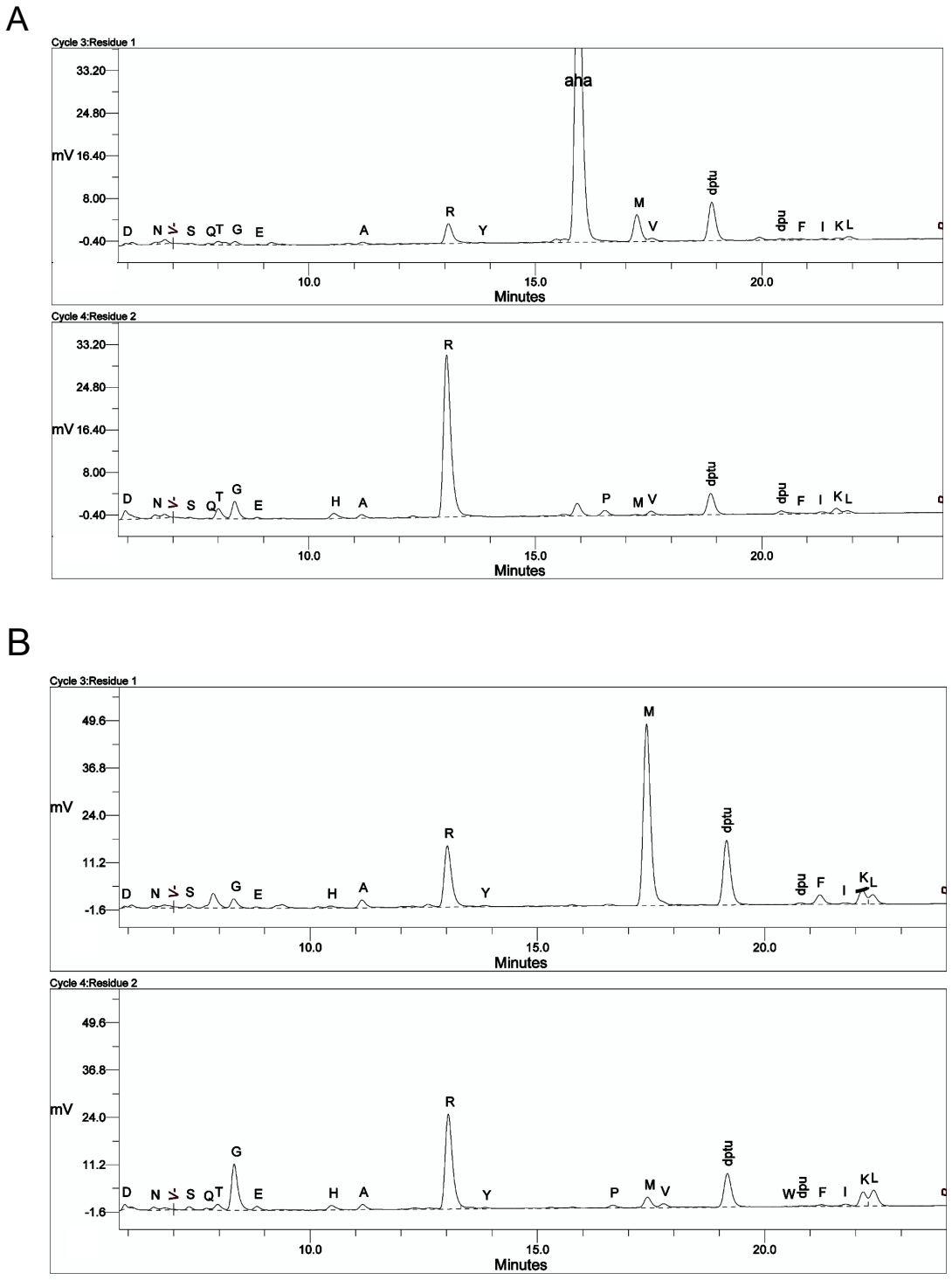

Figure S3. The first two cycles of Edman degradation are shown for (A) a DARPin produced in M9 medium containing Aha, and (B) a DARPin produced in normal 2xYT medium. For the "clickable" DARPin the major sequence is Aha-Arg-Gly-Ser, with a minor fraction of Arg-Gly-Ser as the N-terminus. The retention volume of Aha was distinct from Met (A). For the DARPin expressed in 2YT medium the major sequence was Met-Arg-Gly-Ser, with slightly more observed absence of the initiator methionine (Arg-Gly-Ser), compared to "clickable" DARPins.

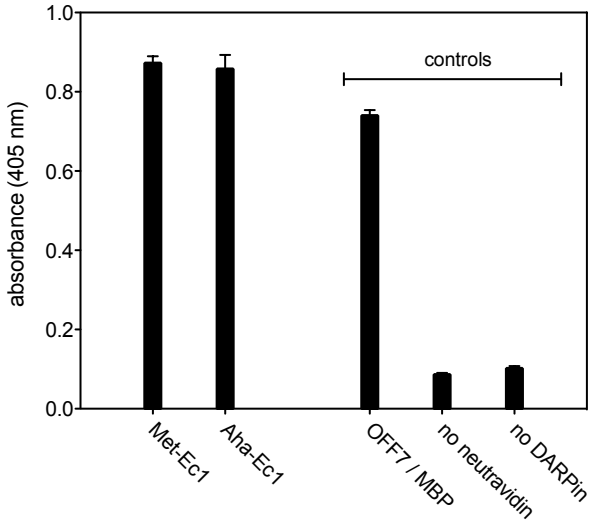

Figure S4. Comparison of Met-Ec1 and Aha-Ec1 by ELISA. The cognate pair of maltose binding protein (MBP) and DARPin OFF7 was used as positive control.

A

sample	Aha-DARPin (μM)	Hpg-DARPin (μM)	Met-DARPin (μM)	$CuSO_4$ (mM)	TCEP (mM)
1	10	10	-	1	2
2	10	10	-	5	2
3	10	-	10	1	2
4	10	-	-	-	-

B

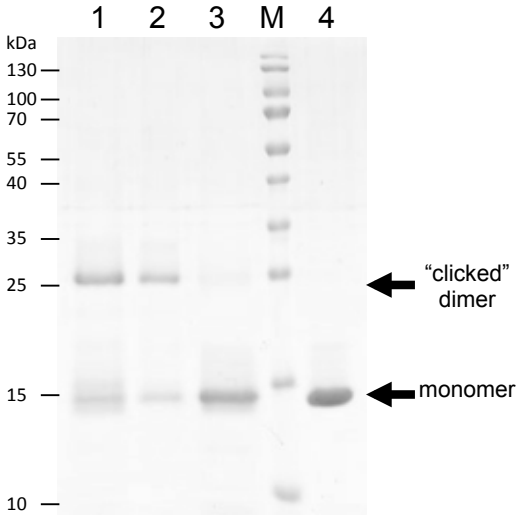

Figure S5. CuAAC linking Hpg- and Aha-modified DARPins to each other. (A) Composition of reaction mixtures and controls. All reactions were carried out in PBS for 3 h at RT. (B) analysis of the reaction mixes by SDS PAGE (15 %).

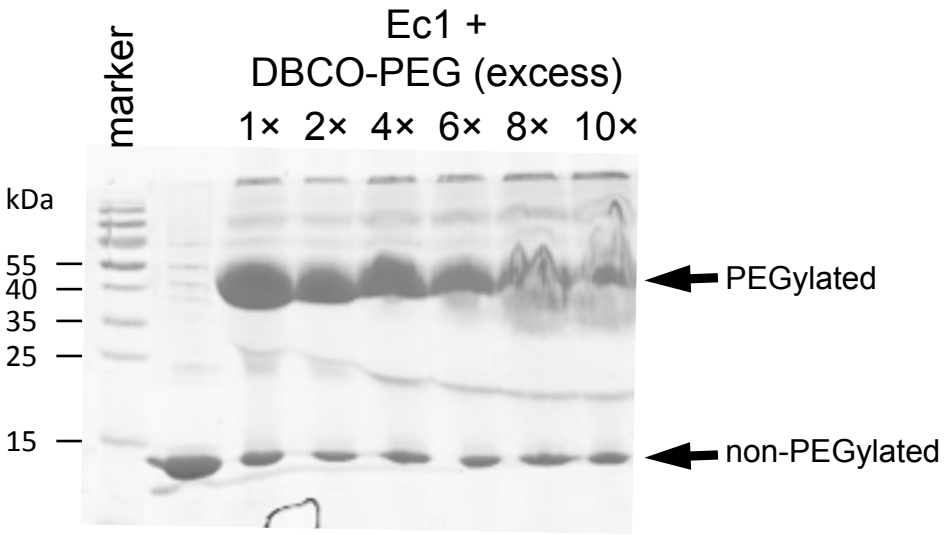

Figure S6. PEGylation of "clickable" Ec1 with increasing molar excess of DBCO-PEG_{20kDa}. Ec1 was at 100 μM during the reaction. The reaction mixture was analyzed after conjugation overnight at 4 °C.

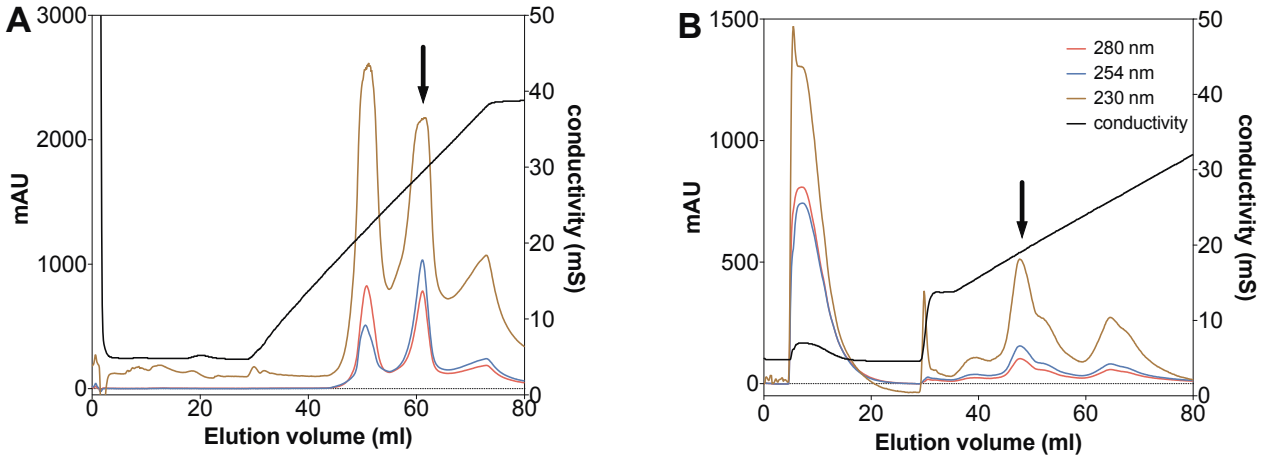

Figure S7. Consecutive anion exchange chromatography elution profiles after labeling with Alexa488-C5-maleimide and after subsequent PEGylation. (A) Separation of Ec1-Alexa488 after conjugation via thiol chemistry. The peak of Alexa488-labelled "clickable" Ec1 is indicated (arrow). It elutes after the unlabeled protein because of the additional negative charge of Alexa488. (B) Elution profile of the PEGylation reaction mix of "clickable" Ec1-Alexa488. The resulting conjugate PEG_{20kDa}-Ec1-Alexa488 was separated and pooled (indicated by an arrow). An SDS PAGE is shown in Fig. 4.

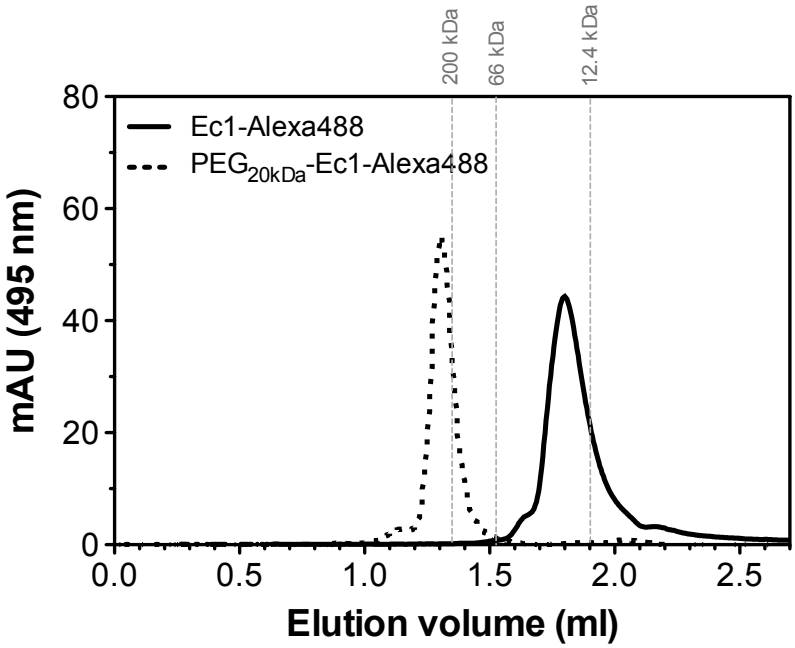

Figure S8. Analytical gel filtration of PEGylated and non-PEGylated Ec1-Alexa488 (Superdex 200 PC3.2). Detection of Alexa488-labeled DARPins at 495 nm.