Supplementary Information

Orthogonal Assembly of a Designed Ankyrin Repeat Proteincytotoxin conjugate with a clickable serum albumin module for half-life extension

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SI Figure S1. SDS-PAGE (15%) analysis of clickable DARPin *Az*Ec1c after expression in *E. coli* and IMAC purification. Protein conjugates were separated under reducing (Red.) or non-reducing (Non-Red.) conditions.



SI Figure S2. Detection of the quantitative conjugation of *Az*Ec1c to mcMMAF by 22% SDS-PAGE. *Az*Ec1c (at a concentration of 384 μ M in PBS) was reacted with a 2-fold molar excess of mcMMAF and the crude reaction mixture was directly assessed by SDS-PAGE to detect the product *Az*Ec1-MMAF and potential side products.



SI Figure S3. Elution profile of *Az*Ec1-MMAF subjected to anion exchange chromatography for removal of unreacted DARPin *Az*Ec1c.



SI Figure S4. Analysis of purified *Az*Ec1-MMAF by SDS-PAGE (22%) and ESI-MS.



	IC ₅₀ (nM)
Ec1	n.d.
MMAF	6
AzEc1-MMAF	0.6
<i>Az</i> Off7∆M-MMAF	436
AzEc1-MMAF + 10× Ec1	231

SI Figure S5. Cytotoxicity of the anti-EpCAM conjugate *Az*Ec1-MMAF and the control conjugate *Az*Off7 Δ M-MMAF against EpCAM-positive HT29 cells. Cells were challenged with various concentrations of the conjugates for 3 days at 37°C before cell viability was determined in colorimetric cell viability assays (XTT). In the competition binding experiment, cells were incubated with saturating concentrations of DARPin Ec1 prior to addition of the conjugate. Each data point represents the mean of triplicates ± SD. All data, with the exception of the unmodified DARPin Ec1, were fitted by a non-linear regression curve (inhibitory dose response with a variable slope model provided by Prism 5, cf. eq 1 in the main manuscript). The table depicts the IC₅₀ values extrapolated from the fit. *n.d.* = no IC₅₀ value could be determined for unmodified Ec1 in the dose range tested.



SI Figure S6. Reaction kinetics of sulfhydryl-activated MSA at 100 μ M with 2.0 eq DBCO-PEG₄-Mal in PBS at 37°C. The conversion to DBCO-modified MSA was followed by RP-HPLC using the DBCO-specific absorption at 309 nm. Samples were taken from the reaction mixture after 5, 10, 15, 20 and 30 min, and the educt DBCO-PEG₄-Mal was subsequently quantified by RP-HPLC. A separate run of DBCO-PEG₄-Mal (at the starting concentration of 200 μ M) was used to quantify the approximate equivalents reacted to form MSA-DBCO.



SI Figure S7. Determination of any hypothetical non-specific interaction of DBCO with serum albumin by RP-HPLC. To test whether an undesired maleimide-independent reaction of MSA with the DBCO core is possible, DBCO-NH₂ (missing a thiol-reactive maleimide) was added in different stoichiometries (0.5 - 3.0 eq) to MSA (100 μ M in PBS) and incubated for 1 h at 37°C. (A) Chromatograms of RP-HPLC runs from each reaction mix detected with the DBCO-specific absorption at 309 nm. (B) Peak areas of non-reacted DBCO-NH₂ were plotted against the appropriate equivalents of DBCO-NH₂ used in the reaction and fitted by linear regression, indicating no significant reaction of the cyclooctyne DBCO with MSA at a concentration of 100 μ M.



SI Figure S8. SDS-PAGE (4-12%) analysis of the SPAAC reaction between AzEc1-MMAF (189 μ M) with MSA-DBCO (1.3 eq) after 72 h at 4°C.



SI Figure S9. Elution profiles from the purification of MSA-Ec1-MMAF by (A) anion exchange chromatography and (B) subsequent preparative gel filtration.



SI Figure S10. Mass determination of unmodified MSA by ESI-MS



SI Figure S11. Mass determination of purified MSA-Ec1-MMAF by ESI-MS.



SI Figure S12. Preparation of Alexa488-labeled conjugates used for cell-binding studies by flow cytometry. SDS-PAGE (10%) was used to monitor crude mixtures after reaction of (A) MSA-DBCO (80-90 μ M) with 1.2 eq *Az*Ec1-Alexa488 or with (B) 1.2 eq *Az*Off7 Δ M-Alexa488 for 72 h at 4°C.



SI Figure S13. Chromatograms from (A, C) anion exchange and (B, D) preparative size exclusion chromatography runs for the purification of Alexa488-labeled conjugates for flow cytometry.



SI Figure S14. Analysis of purified MSA-Ec1-Alexa488 and MSA-Off7∆M-Alexa488, and their building blocks by SDS-PAGE (4-12%).



SI Figure S15. Determination of association and dissociation rate constants of (A) unmodified DARPin Ec1, and the conjugates (B) *Az*Ec1-MMAF and (C) MSA-Ec1-MMAF by surface plasmon resonance (ProteOn XPR36, Bio-Rad) using a NLC chip coated with the extracellular domain of human EpCAM (hEpEX).



SI Figure S16. Serum stability of MSA-Ec1-MMAF. The conjugate was diluted in nonheat activated mouse serum at a concentration of 1 μ M and incubated at 37°C before aliquots were withdrawn at different time points. Samples were subjected to pull-down of His-tagged conjugates by magnetic Ni-NTA agarose beads, separated on SDS-PAGE and subsequently detected by Western blotting, using an anti-RGSHis₆-HRP Ab conjugate for direct detection of the N-terminal RGSHis-tag of DARPins.