SUPPLEMENTARY DATA

Increasing the anti-tumor effect of an EpCAM-targeting fusion toxin by facile click PEGylation

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Supplementary Tables

Table S1. Affinities of Ec1-ETA", Aha-Ec1-ETA" and PEG_{20kDa}-Ec1-ETA" determined by surface plasmon resonance (SPR) on immobilized EpCAM (sensograms are shown in Suppl. Fig. S4).

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	R_{max} (RU)	K_D (pM)
Ec1-ETA"	3.1×10^4	5.1×10^{-6}	515	165
Aha-Ec1-ETA"	3.0×10^4	4.1×10^{-6}	481	139
PEG_{20kDa}-Ec1-ETA"	1.6×10^4	4.7×10^{-6}	210	290

Table S2. Cytotoxicity of fusion toxins against EpCAM-positive tumor cell lines.

Cell line	Ec1-ETA" (mol/l)	PEG_{20kDa}-Ec1-ETA" (mol/l)	Fold difference
HT29	5.1×10^{-14}	5.1×10^{-13}	10
MDA-MB-468	4.2×10^{-13}	3.4×10^{-12}	8
MCF7	5.9×10^{-14}	2.5×10^{-13}	4

Cytotoxicity was determined in 96 h XTT assays, and viability curves were fitted by non-linear regression and used for calculation of the IC_{50}. The difference in potency between Ec1-ETA" and PEG_{20kDa}-Ec1-ETA" was calculated for each cell line for comparison.

Supplementary Figures

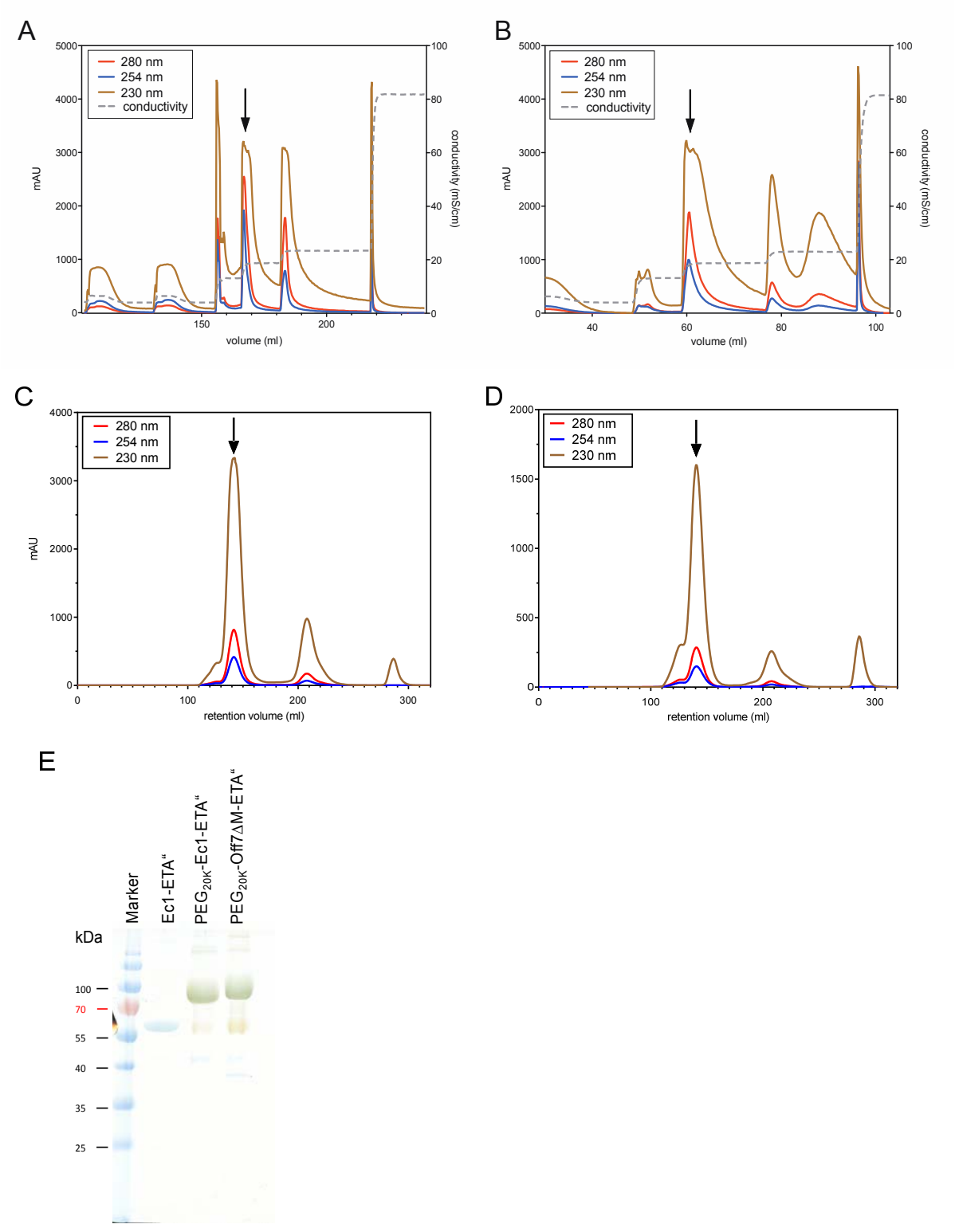

Figure S1. Purification of PEGylated ETA" fusion toxins by anion exchange chromatography (A, B) followed by preparative size exclusion chromatography (C, D). The elution profiles of PEG_{20kDa}-Ec1-ETA" (A, C) and the non-targeted control fusion toxin PEG_{20kDa}-Off7ΔM-ETA" (B, C) are shown. SDS PAGE analysis of PEGylated fusion toxins, and detection by Arrows indicate the desired products as verified by SDS PAGE. (E) Coomassie staining and PEG staining with iodine Monomeric fractions of either PEGylated protein were pooled and used for further studies.

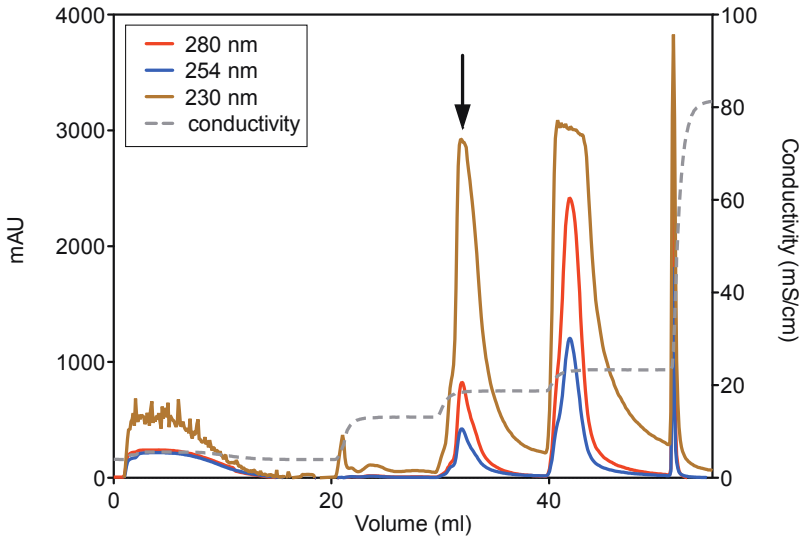

Figure S2. Anion exchange chromatography of fusion toxin, which remained non-PEGylated after a first round of modification and was then recycled to react again with DBCO-PEG_{20kDa}. Non-PEGylated Ec1-ETA" was recovered from previous chromatography runs, concentrated and again used for PEGylation. The chemical reactivity of the azide-containing fusion toxin was maintained after the first round, finally resulting in an increased PEGylation yield. The arrow indicates the desired product, as verified by SDS PAGE (not shown).

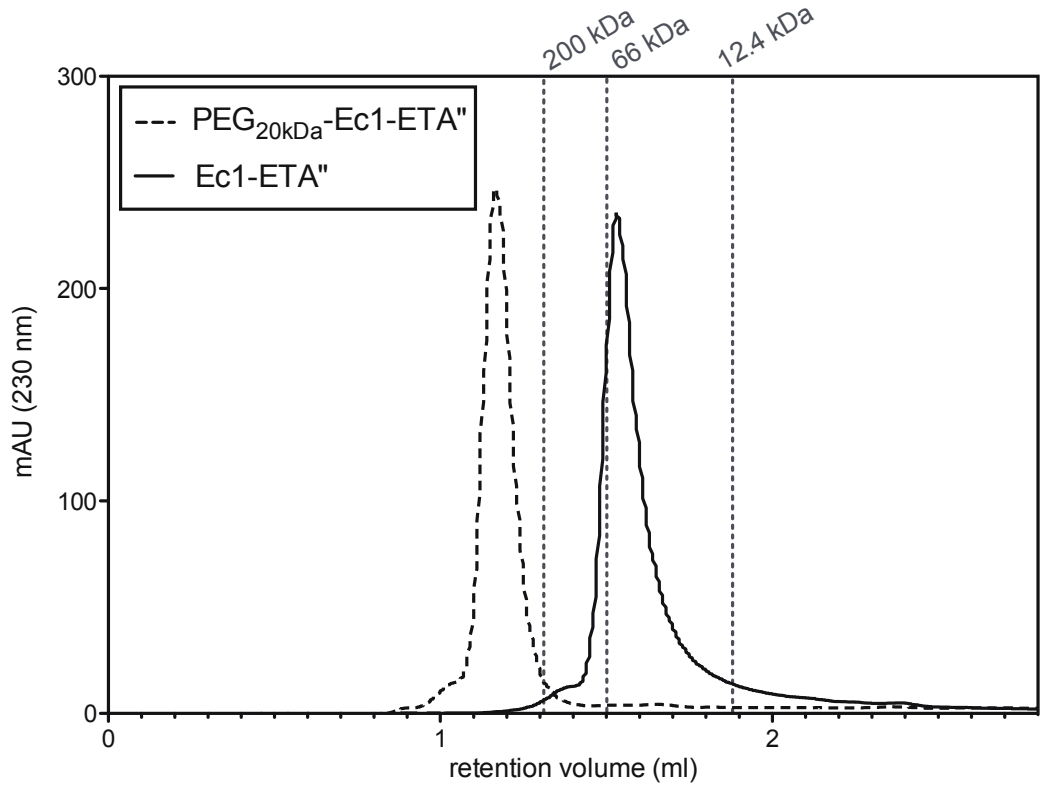

Figure S3. Comparison of Ec1-ETA" and PEG_{20kDa}-Ec1-ETA" by analytical size exclusion chromatography (aSEC). PEGylation lead to a massive increase in the hydrodynamic radii of the fusion toxins changing the elution profile to smaller elution volumes. The nominal molecular weight of Ec1-ETA" is 60 kDa, PEGylation with PEG_{20kDa} increased the apparent molecular weight to approx. 250 kDa.

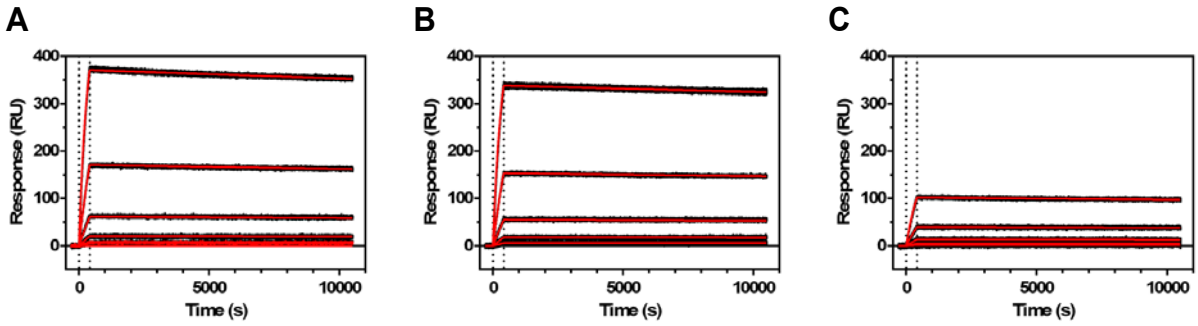

Figure S4. Effect of N-terminal click-PEGylation on the affinity of Ec1-ETA". SPR sensograms of (A) Ec1-ETA", (B) Aha-Ec1-ETA" and (C) PEG_{20kDa}-Ec1-ETA" are shown (the numeric values are summarized in Supplementary Table S1). Different concentrations of the respective fusion toxin (from highest to lowest curve: 100 nM, 31.6 nM, 10 nM, 3.16 nM, 1 nM) were applied. Association and dissociation rate constants were determined from normalized data measured in duplicates by surface plasmon resonance on an EpCAM-immobilized chip. A Langmuir 1:1 model was applied to fit the data.

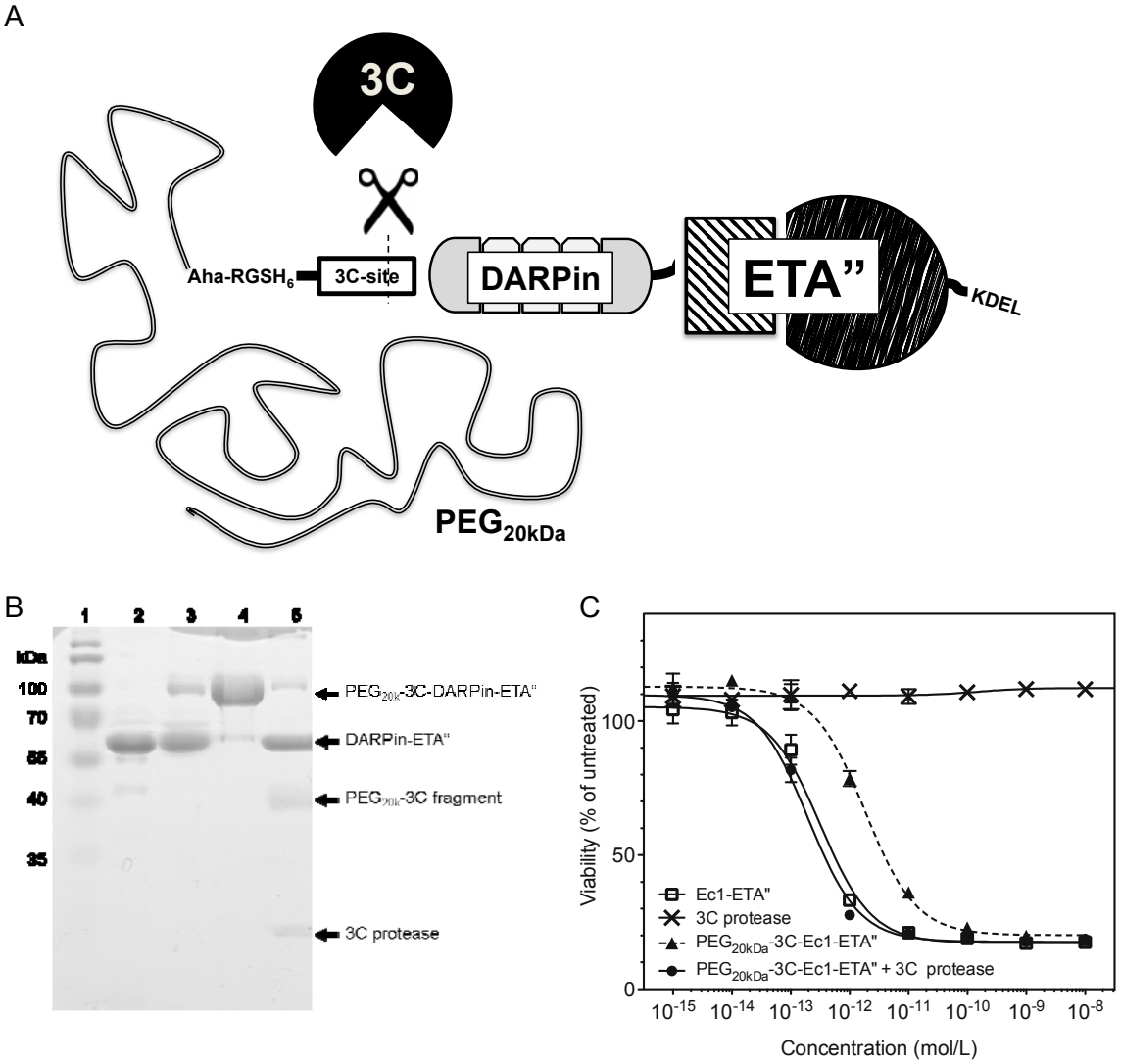

Figure S5. Characterization of PEGylated Ec1-ETA” containing an N-terminal 3C (PreScission) protease site for proteolytic removal of the polymer. (A) Schematic illustration. (B) Quantitative removal of the N-terminal PEG (de-PEGylation) from PEG_{20kDa}-DARPin-ETA” detected by SDS PAGE. Lane 1: PageRuler (Fermentas) marker, lane 2: DARPin-ETA”, lane 3: 3C-DARPin-ETA”, lane 4: PEG_{20kDa}-3C-DARPin-ETA”, lane 5: 3C protease + PEG_{20kDa}-3C-DARPin-ETA”. (C) Restoration of the cytotoxicity of PEG_{20kDa}-Ec1-ETA” after processing with 3C protease. The fusion toxin PEG_{20kDa}-3C-Ec1-ETA” containing an N-terminal cleavage site specific for 3C protease was proteolytically de-PEGylated and examined in 72 h XTT assays using HT29 cells as target. Viability curves were fitted by non-linear regression and used for calculation of the IC_{50} values: 2.0×10^{-13} mol/L for non-PEGylated Ec1-ETA”, 1.8×10^{-12} mol/L for PEGylated Ec1-ETA”, 3.2×10^{-13} mol/L for dePEGylated Ec1-ETA”. Treatment with 3C protease alone did not reduce cell viability in the dose range tested (< 10 nM). Bars = SD.

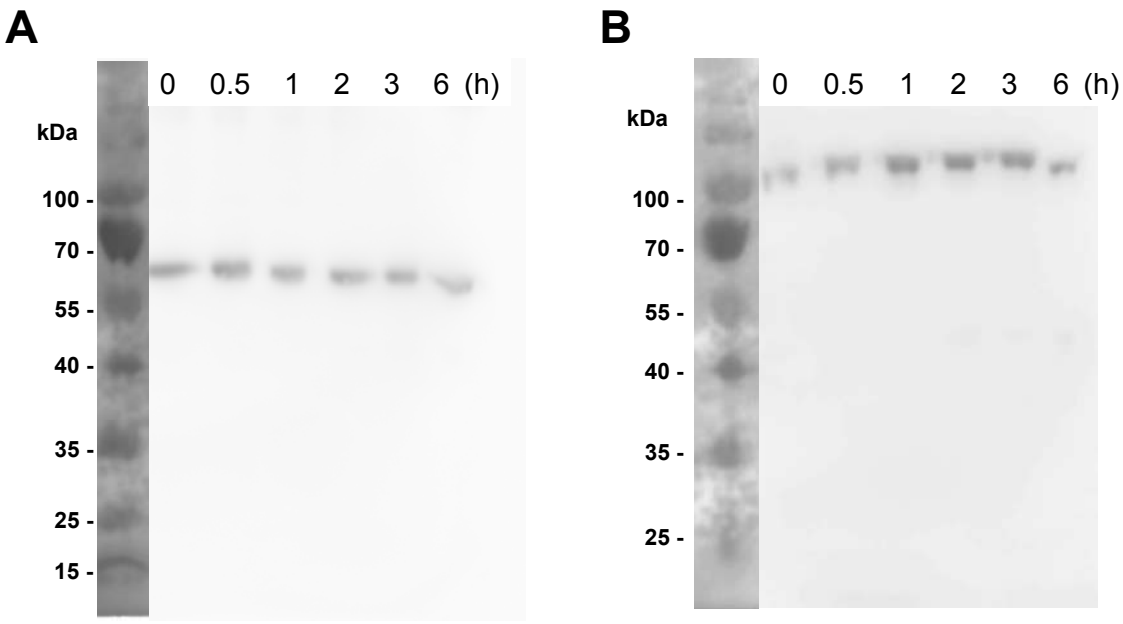

Figure S6. *In vitro* serum stability of fusion toxins. Ec1-ETA" (A) or PEG_{20kDa}-Ec1-ETA" (B) were incubated in triplicates for up to 6 h in mouse serum (at 37 °C) before the integrity of the fusion toxins and the stability of the triazole linkage for the PEGylated variant was assessed by Western blotting. One representative blot is shown.