Supplementary Information for:

Optimizing the anti-tumor efficacy of protein-drug conjugates by engineering of the molecular size and half-life

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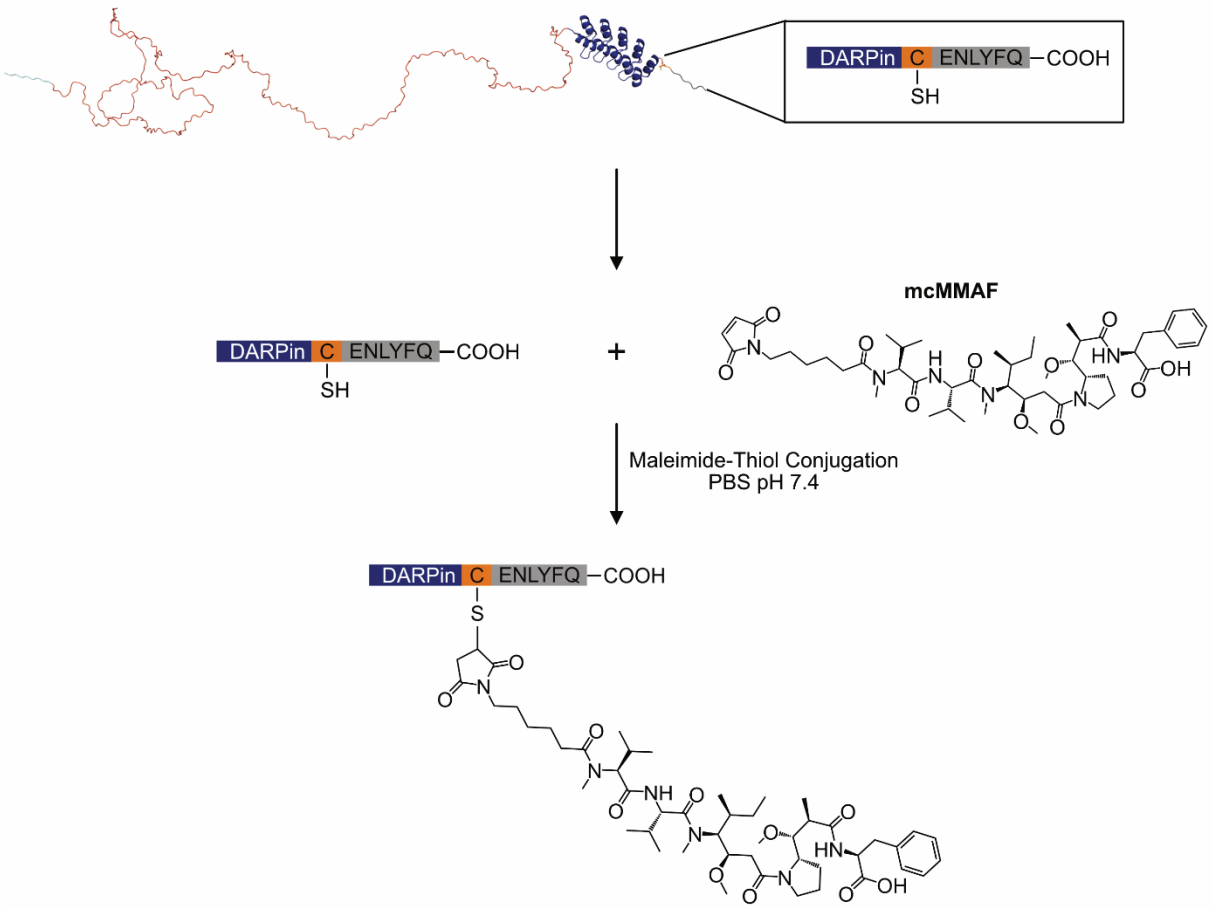


Fig. S1: Schematic illustration of the maleimide-thiol conjugation of maleimidocaproyl monomethyl auristatin F (mcMMAF) to the single C-terminal cysteine of polypeptide-DARPin fusion proteins.

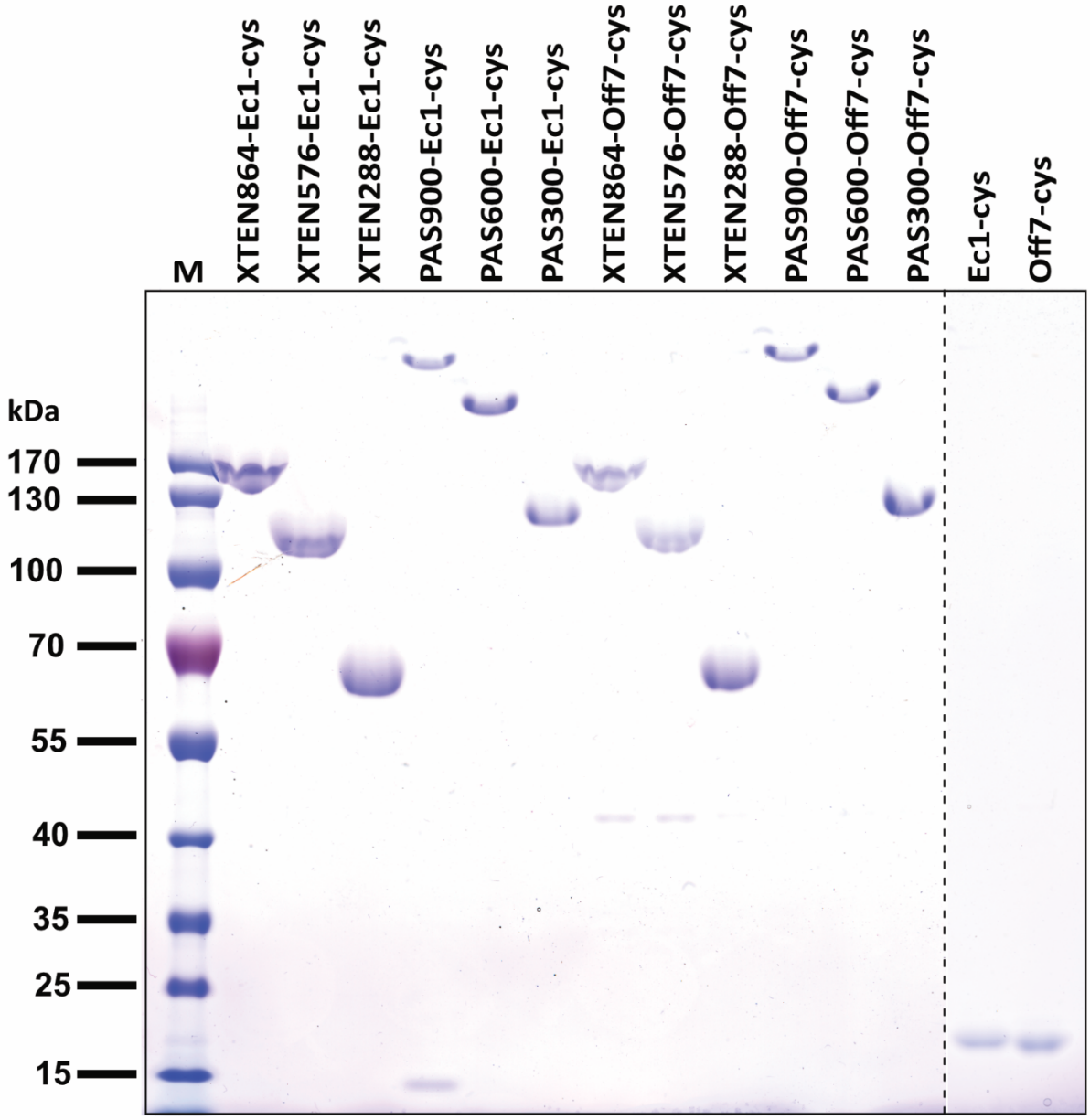


Fig. S2: SDS-PAGE of purified proteins with a single C-terminal cysteine for conjugation with mcMMAF. Proteins (113 pmol) were separated on a 4–12% NuPAGE™ Bis-Tris Protein Gel (Thermo Fisher Scientific) and stained with Coomassie Brilliant Blue R-250.

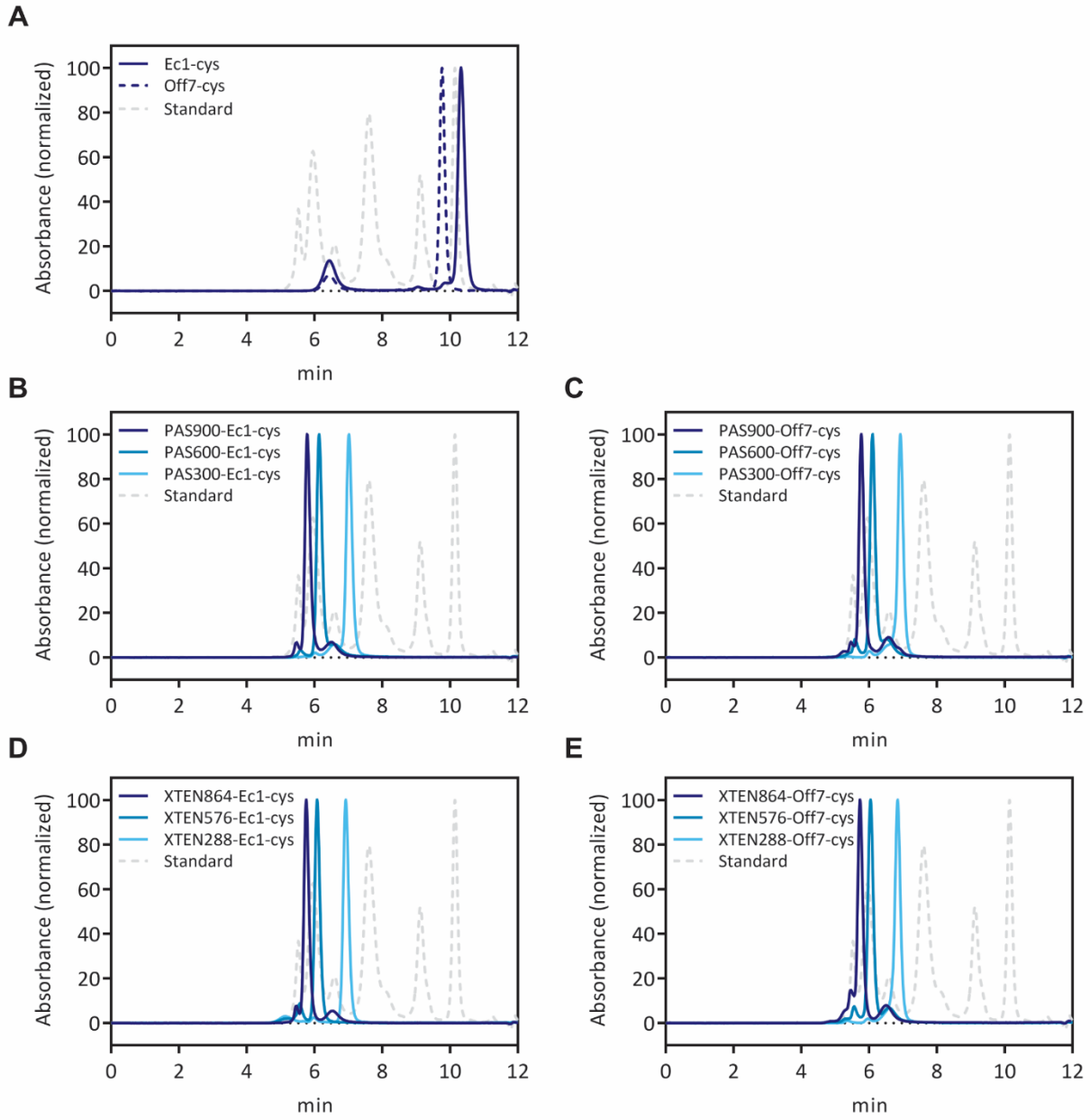


Fig. S3: Analysis of proteins by analytical SEC-HPLC after purification. A) Unmodified DARPins, B) PASylated DARPin Ec1, C) PASylated DARPin Off7, D) XTENylated DARPin Ec1 and E) XTENylated DARPin Off7. Proteins were analyzed on an Agilent Advanced BioSEC column (Agilent) connected to an Agilent 1260 Infinity Bio-inert Quaternary LC HPLC system (Agilent). Ten microliters of 10 µM dilutions in PBS pH 7.4 were injected to the HPLC system at a flow rate of 0.35 mL/min. The elution profiles were monitored by absorbance at 280 nm and 230 nm. For calibration, an injection of high molecular weight gel filtration standard (Agilent AdvanceBio SEC 300Å Protein Standard / PN 5190-9417) was included.

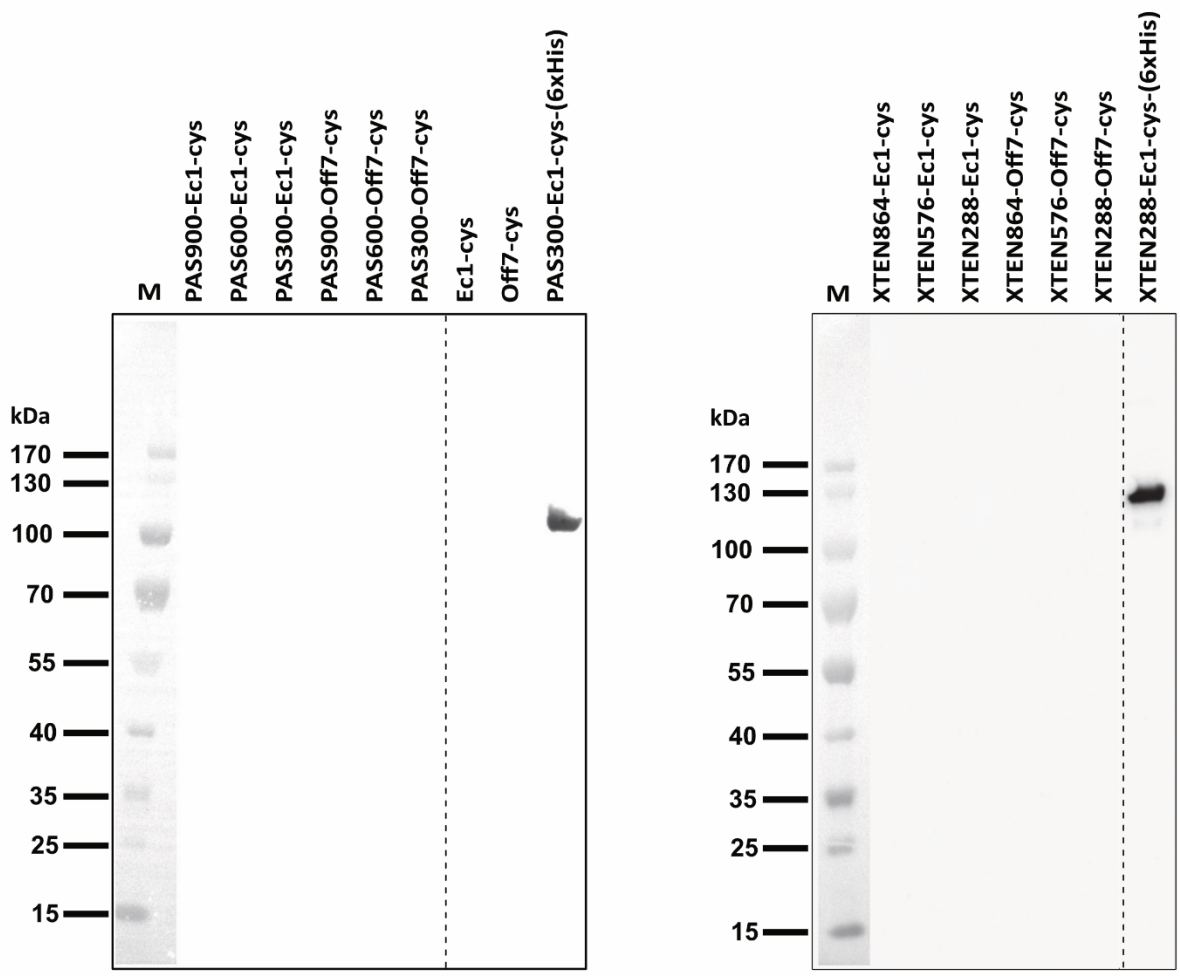


Fig. S4: Detection of C-terminal His_6-tags on DARPin-cys and polypeptide-DARPin-cys fusion proteins for evaluation of the removal of His_6-tags during purification by Western blotting and chemiluminescent immunodetection. Fusion proteins (13 pmol) were separated on a 4–12% NuPAGE™ Bis-Tris Protein Gel (Thermo Fisher Scientific) and stained and blotted to a nitrocellulose membrane. Detection of the C-terminal His_6-tag was carried out with a mouse anti-His_4 primary antibody (Qiagen PN 34670 / 1:1000 dilution). The recorded chemiluminescence signals were generated by substrate conversion of a goat anti-mouse IgG HRP conjugate (Thermo Fisher Scientific PN 31438 / 1:5000). The quantitative removal of the His_6-tag is apparent, except in the uncleaved control in the rightmost lane.

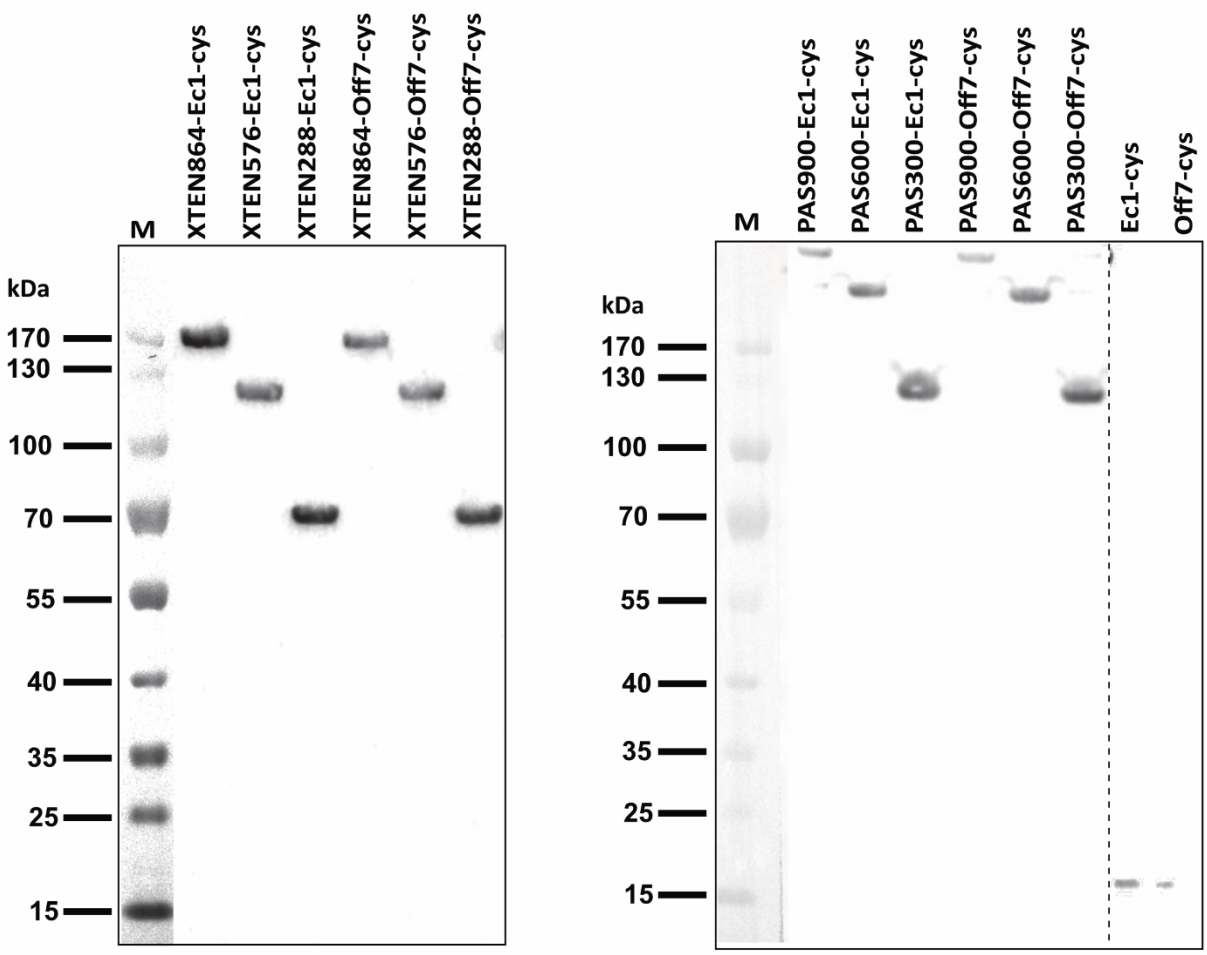


Fig. S5: Detection of N-terminal FLAG-tags on DARPin-cys and polypeptide-DARPin-cys fusion proteins for evaluation of purity by Western blotting and chemiluminescent immunodetection. Fusion proteins (13 pmol) were separated on a 4–12% NuPAGE™ Bis-Tris Protein Gel (Thermo Fisher Scientific) and stained and blotted to a nitrocellulose membrane. The N-terminal FLAG-tag was detected with a mouse anti-FLAG M2 primary antibody. The recorded chemiluminescence signals were generated by substrate conversion of a goat anti-mouse IgG HRP conjugate.

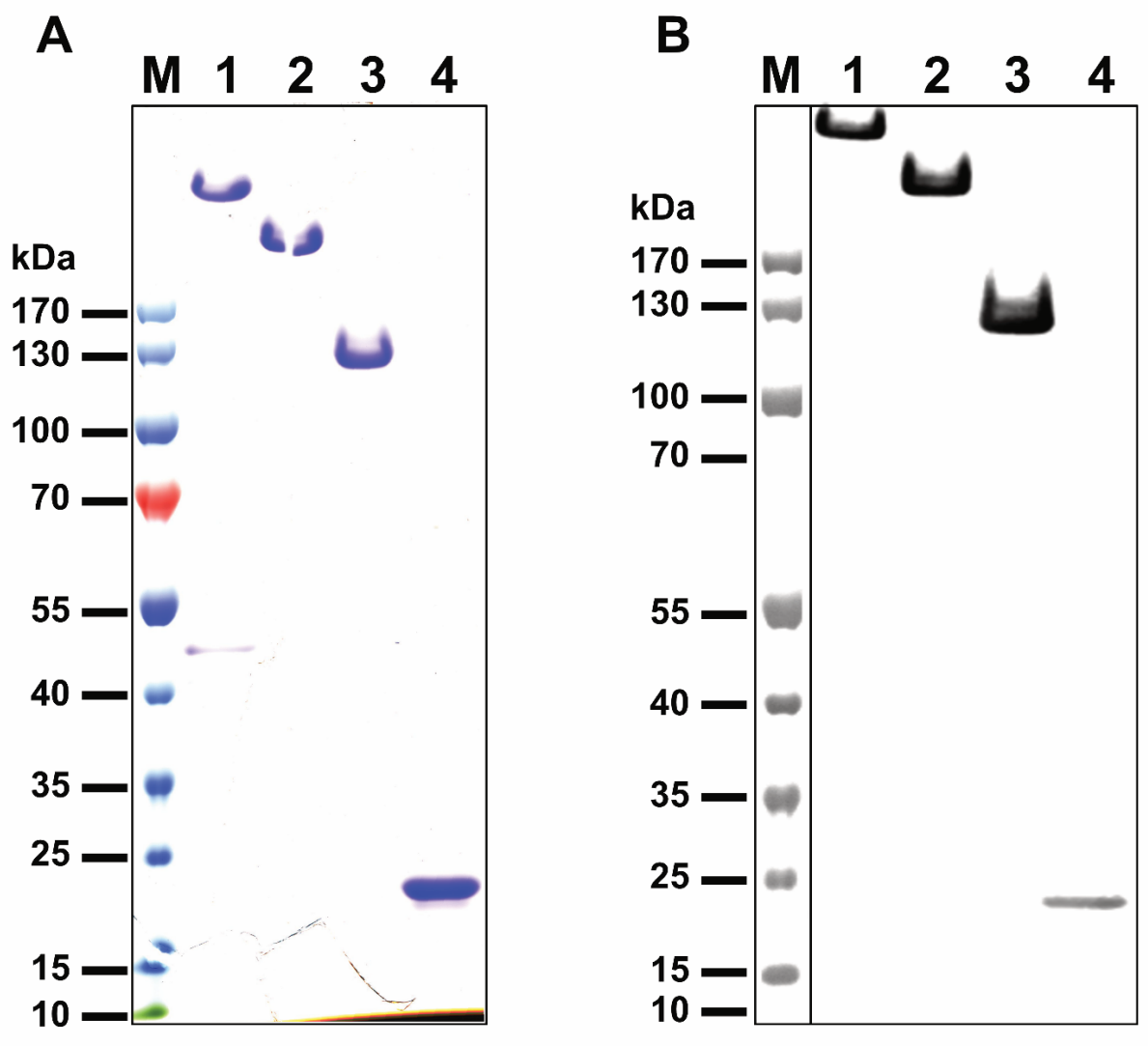


Fig. S6: Analysis of protein-MMAF conjugate purity by SDS-PAGE and Western blotting. A) SDS-PAGE of purified protein MMAF-conjugates. A) MMAF-conjugates (113 pmol) were separated on a 4–12% NuPAGE™ Bis-Tris Protein Gel (Thermo Fisher Scientific) and stained with Coomassie. B) MMAF-conjugates (13 pmol) were separated on a 4–12% NuPAGE™ Bis-Tris Protein Gel (Thermo Fisher Scientific) and blotted to a nitrocellulose membrane. The N-terminal FLAG-tag was detected with a mouse anti-FLAG M2 primary antibody. The recorded chemiluminescence signals were generated by substrate conversion of a goat anti-mouse IgG HRP conjugate. Lane: 1) PAS900-Off7-MMAF, 2) PAS600-Off7-MMAF, 3) PAS300-Off7-MMAF, 4) Off7-MMAF.

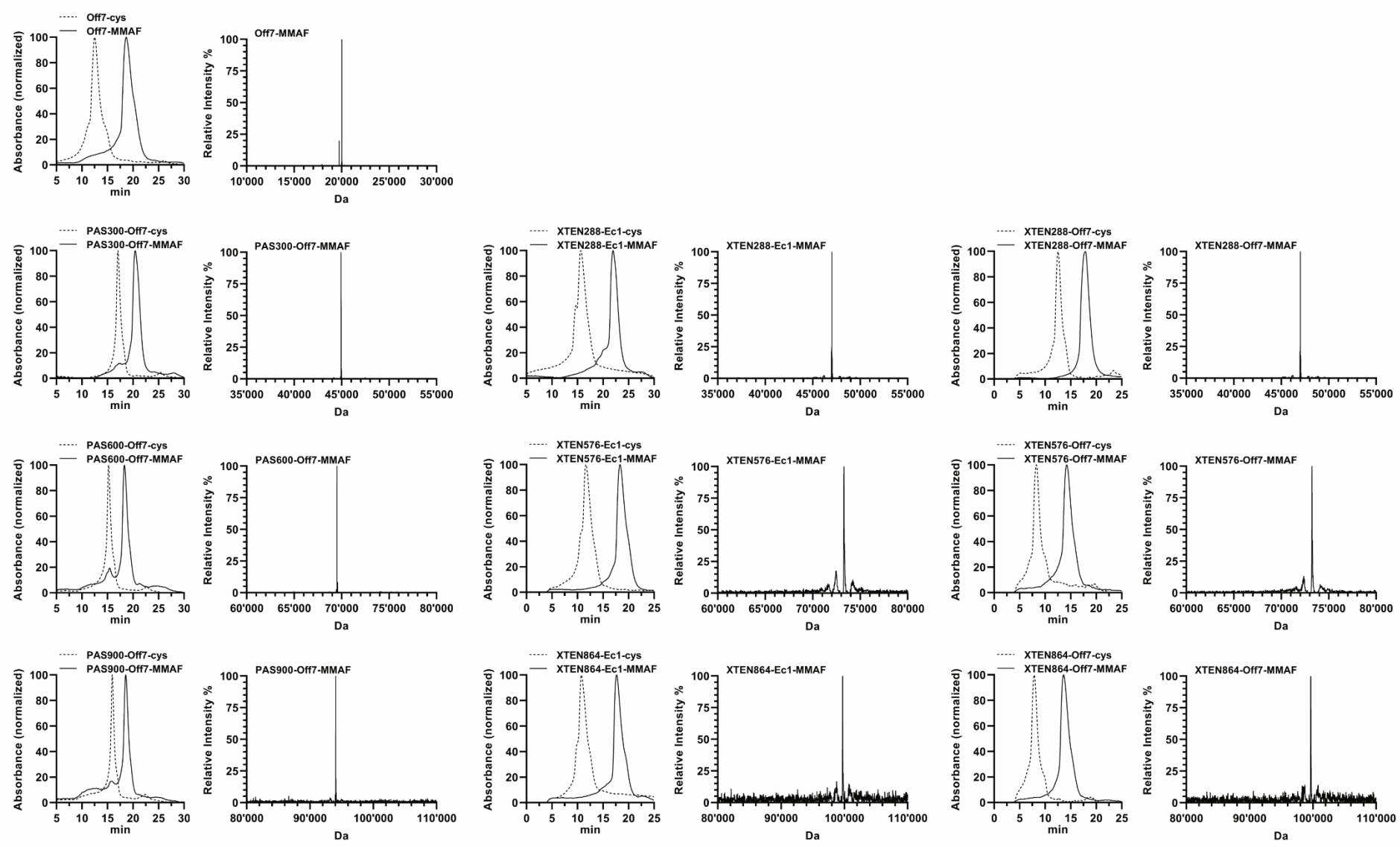


Fig. S7: Analysis of protein-MMAF conjugate purity by analytical hydrophobic interaction chromatography (HIC) and electrospray ionization mass spectroscopy (ESI-MS). The values are shown in Table S1

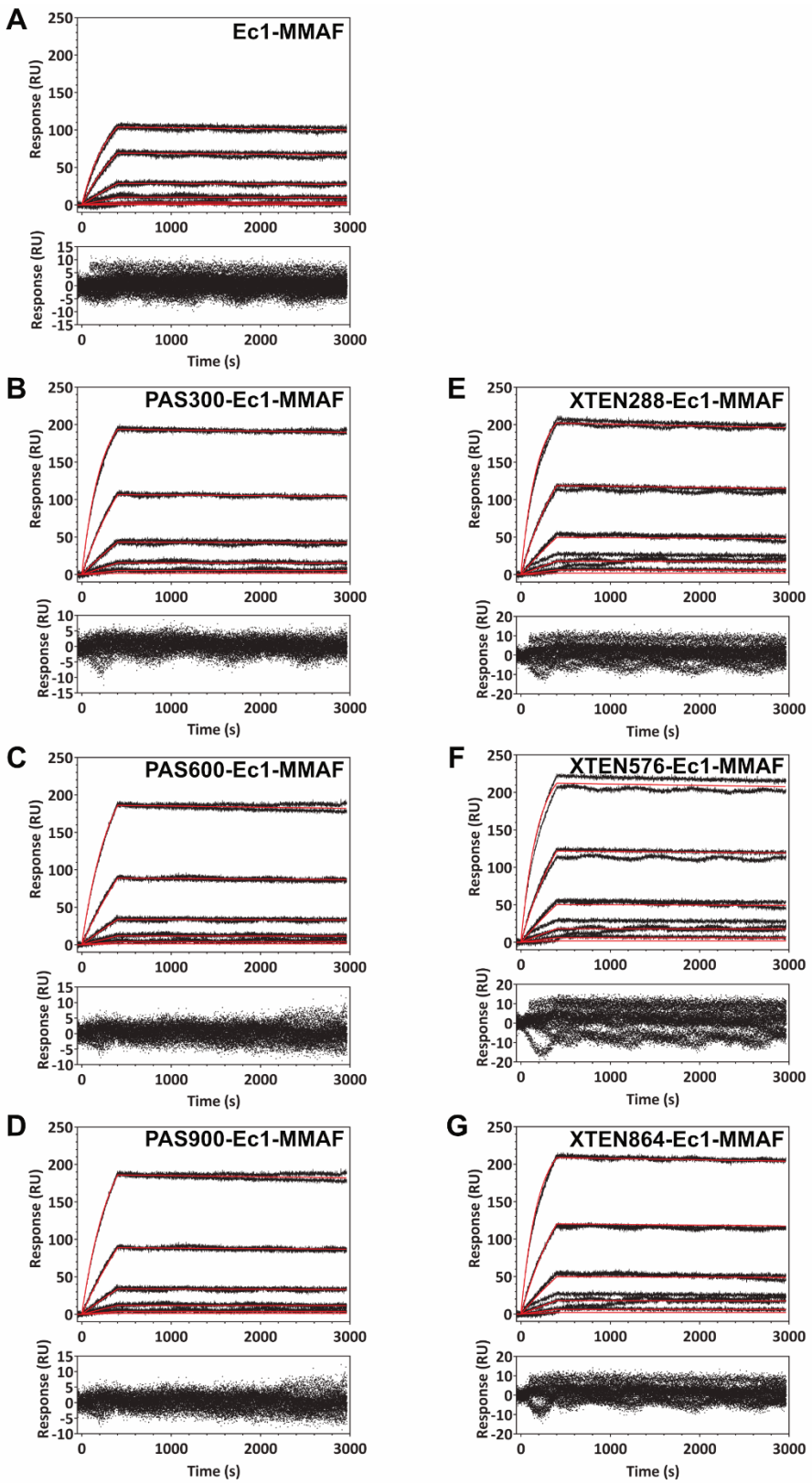


Fig. S8: Binding kinetics of polypeptide-Ec1-MMAF and Ec1-MMAF conjugates measured by SPR. The biotinylated extracellular domain of human EpCAM (bioEpEx) was immobilized on the chip surface. Serial dilutions (0.33, 1, 3, 9, 27, 81 nM) of the MMAF conjugates were injected at a flow rate of 60 µL/min. The ligand coated surface was regenerated by short pulses of 100 mM phosphoric acid after every analyte injection. Sensorgrams were fitted with a 1:1 Langmuir binding model to determine the kinetic parameters k_a, k_d and K_D. Injection replicates are shown in black, fits in red. A) Ec1-MMAF (K_D = 64.8 pM) B) PAS300-Ec1-MMAF (K_D = 141 pM) C) PAS600-Ec1-MMAF (K_D = 262 pM) D) PAS900-Ec1-MMAF (K_D = 219 pM) E) XTEN288-Ec1-MMAF (K_D = 183 pM) F) XTEN576-Ec1-MMAF (K_D = 131 pM) G) XTEN864-Ec1-MMAF (K_D = 134 pM).

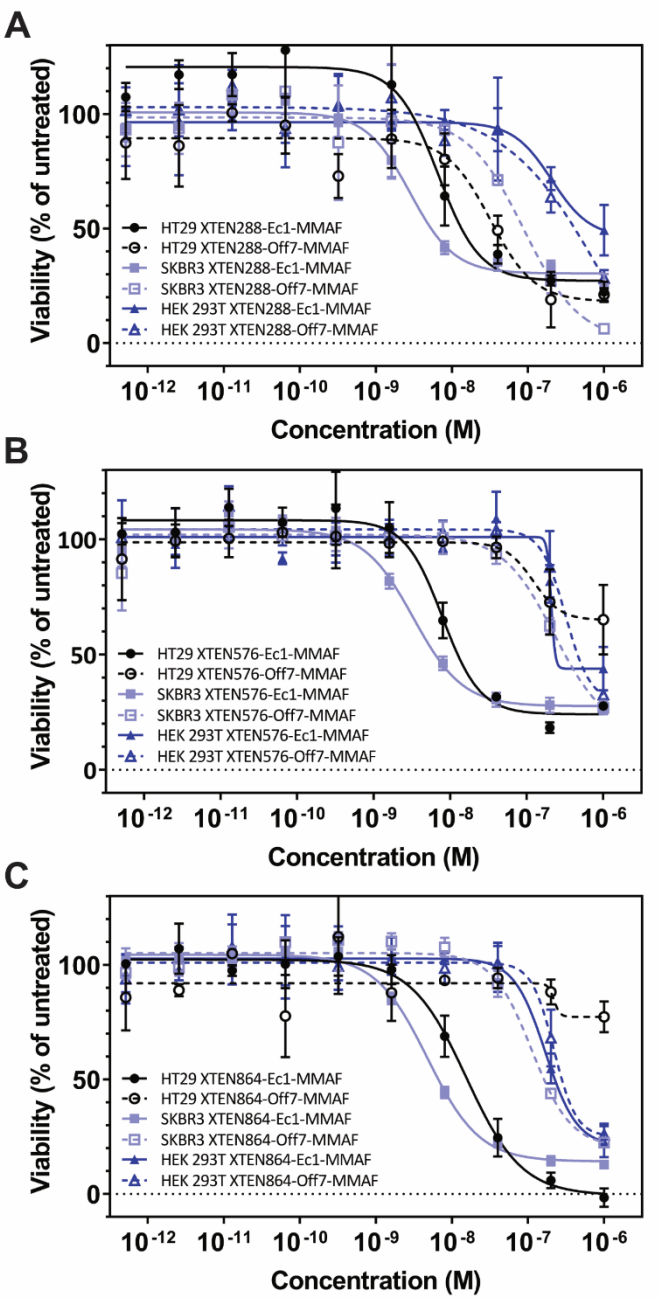
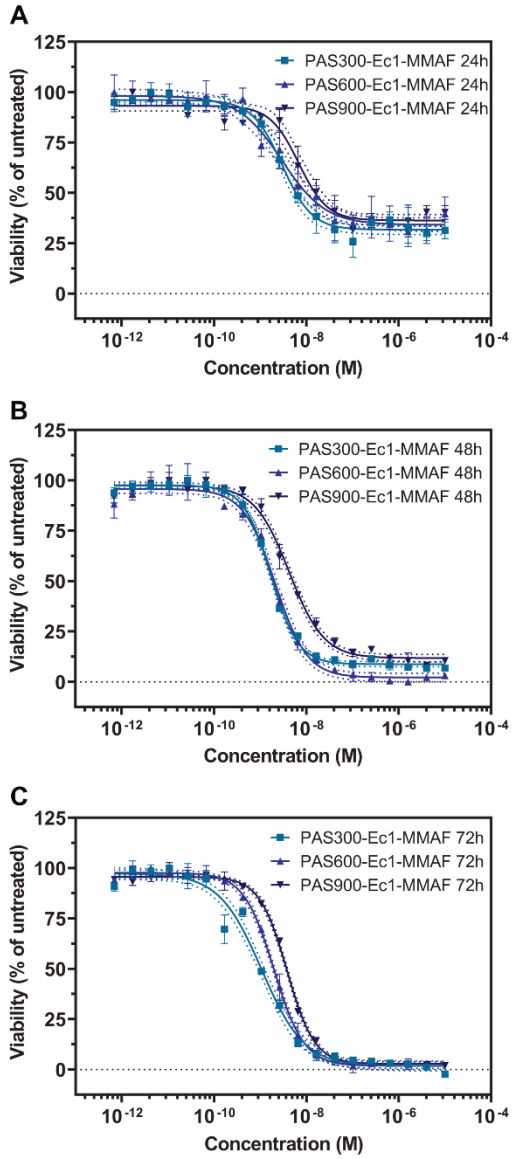


Fig. S9: Cytotoxicity of XTEN-DARPin-MMAF conjugates determined in XTT cell viability assays. The EpCAM specific XTEN-Ec1-MMAF conjugates were compared to the control conjugates XTEN-Off7-MMAF. EpCAM-positive cell lines were A) HT29 and B) SKBR3, in C) EpCAM-negative HEK293T cells were used as control. Cells were incubated with a serial dilution of the MMAF conjugates and free MMAF for 72 h before cytotoxicity was determined. Each data point corresponds to the mean of triplicate measurements ± SD.



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95% CI of Bottom Plateau (Normalized Cell Viability %)	PAS300-Ec1-MMAF	PAS600-Ec1-MMAF	PAS900-Ec1-MMAF
24 h	29.3 - 34.0	30.6 - 37.5	33.3 - 39.3
48 h	7.5 - 10.0	-0.0 - 4.3	9.7 - 13.6
72 h	-0.8 - 4.2	0.5 - 3.1	1.7 - 3.9

95% CI of IC_{50} (nM)	PAS300-Ec1-MMAF	PAS600-Ec1-MMAF	PAS900-Ec1-MMAF
24 h	2.4 - 3.7	2.0 - 4.3	5.1 - 9.0
48 h	1.6 - 1.8	1.8 - 2.4	3.7 - 4.8
72 h	0.9 - 1.3	1.8 - 2.1	3.5 - 4.0

Fig. S10: Cytotoxicity of PAS-DARPin-MMAF conjugates against EpCAM-positive HT29 cells as a function of time determined in XTT cell viability assays. The assays were developed after 24 h (A), 48 h (B) and 72 h (C). Each data point corresponds to the mean of triplicate measurements ± SD. The dashed lines correspond to the 95% confidence intervals (95% CI) of the fits. D) Comparison of the 95% CI bottom plateaus of the fits and 95% CI of the IC_{50} concentrations for time-dependent comparison.

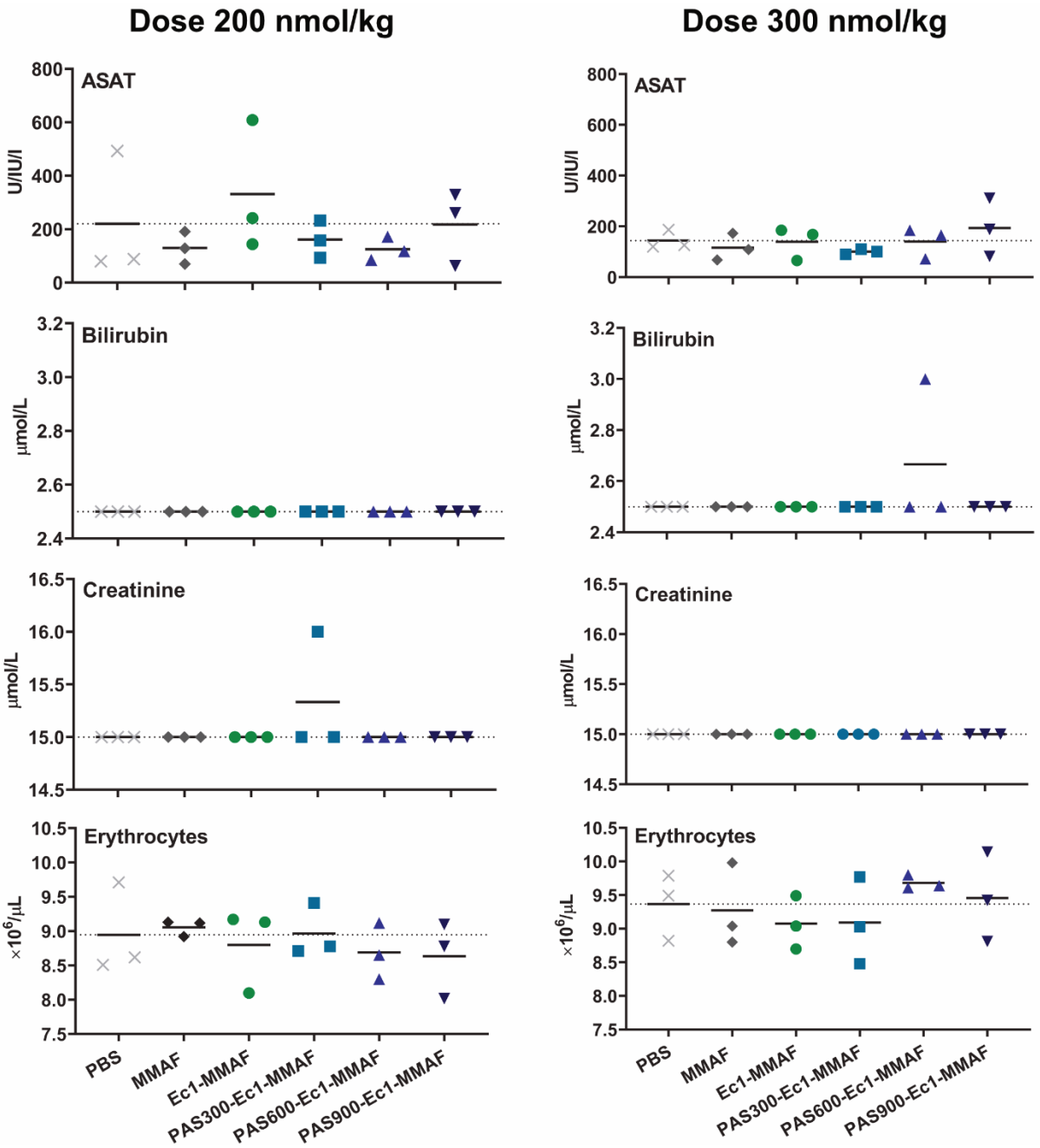

Fig. S11: Tolerability of Ec1-MMAF and PAS-Ec1-MMAF conjugates in CD-1 nude mice. Conjugates were injected every other day for five times and the body weight was monitored daily. One week after the last injection, mice were euthanized and the blood was analyzed for levels of aspartate aminotransferase activity (ASAT), bilirubin, creatinine and erythrocytes. For each group 3 mice were analyzed.

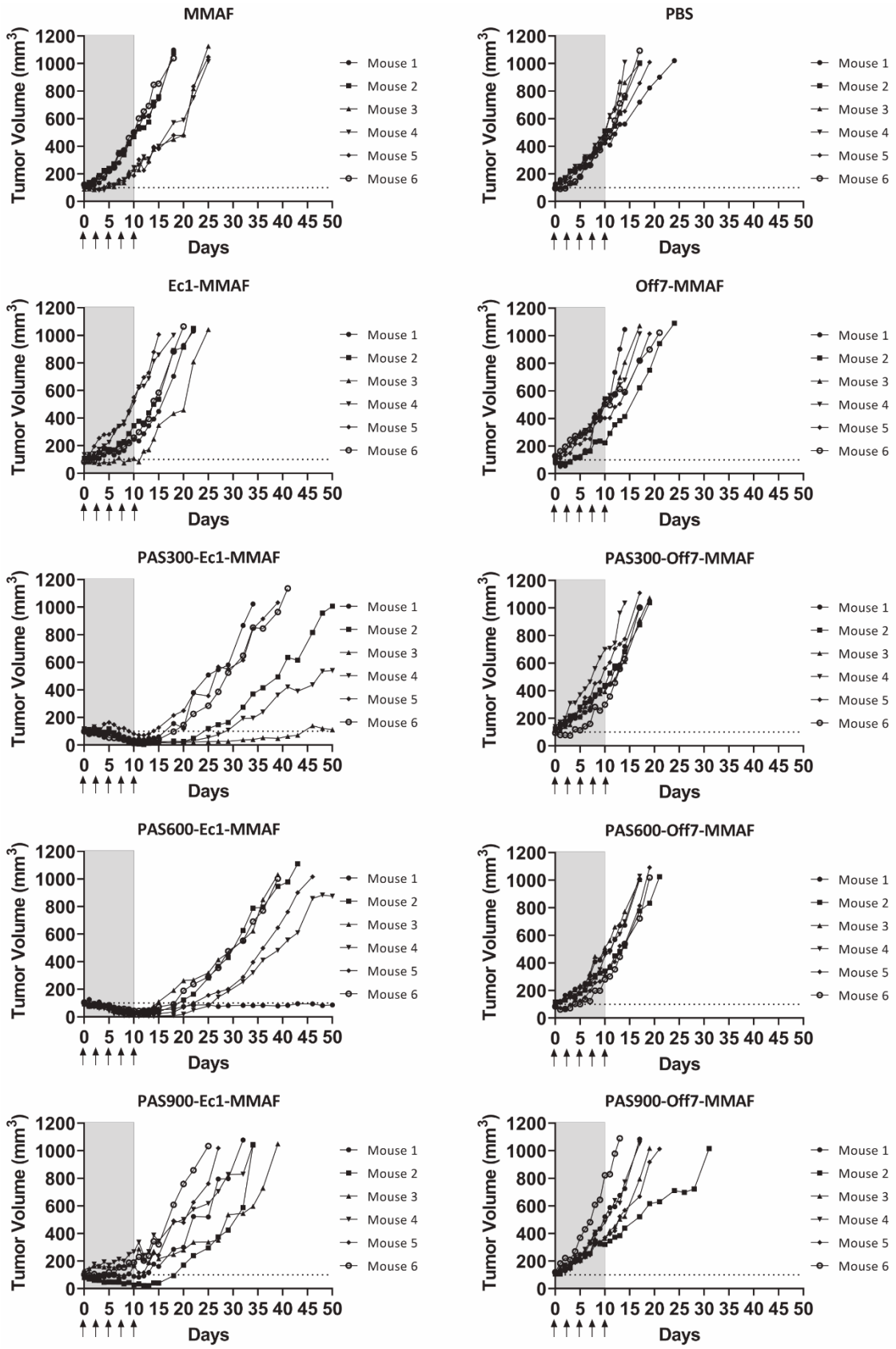

Fig. S12: Anti-tumor effect of DARPin-MMAF and PAS-DARPin-MMAF conjugates in CD-1 nude mice. Per treatment group six mice bearing EpCAM-positive HT29 tumor xenografts were injected every other day for five times with equimolar doses (300 nmol/kg) of free MMAF, protein-MMAF conjugate or 150 µL PBS. Arrows indicate days of injection. Tumor growth was monitored by caliper measurement during the course of 50 days and is shown here individually for each single mouse in the treatment group. Data points represent the tumor volume of a single mouse.

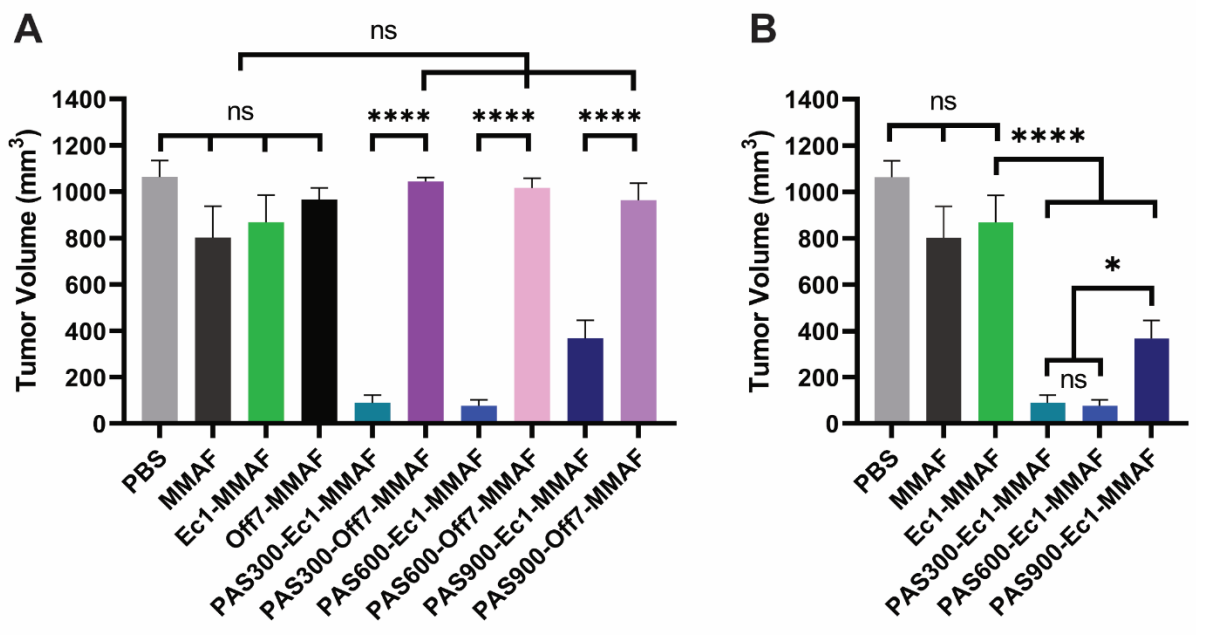

Fig. S13: Statistical analysis of differences in tumor volume at day 18 after the start of treatment. Statistical analysis was performed by one-way ANOVA using GraphPad Prism; ns = P > 0.05, * = P < 0.05, **** = P < 0.0001. A) Pair-wise comparison of size-matched treatment groups. B) Comparison of half-life extended EpCAM-targeting conjugates vs. non-half-life extended conjugates, and of free MMAF and vehicle treated groups.

Table S1: Biophysical characterization of PASylated and XTENylated and unmodified EpCAM-targeting DARPin Ec1 and control DARPin Off7.

Construct	**MW_{calc}[a] (kDa)**	**MW_{sxp_MS}[b] (kDa)**	**Purity[c] (%)**	**k_a[d] ($M^{-1}s^{-1}$)**	**k_d[d] (s^{-1})**	**K_D[d] (M)**
Off7-MMAF	20.018	20.018	>95%	N.A.	N.A.	N.A.
PAS300-Off7-MMAF	44.942	44.942	>95%	N.A.	N.A.	N.A.
PAS600-Off7-MMAF	69.507	69.507	>95%	N.A.	N.A.	N.A.
PAS900-Off7-MMAF	94.073	94.073	>95%	N.A.	N.A.	N.A.
XTEN288-Ec1-MMAF	47.013	47.012	>95%	6.84×10^{4}	1.25×10^{-5}	1.83×10^{-10}
XTEN576-Ec1-MMAF	73.295	73.296	>95%	6.48×10^{4}	8.49×10^{-6}	1.31×10^{-10}
XTEN864-Ec1-MMAF	99.721	99.727	>95%	6.54×10^{4}	8.78×10^{-6}	1.34×10^{-10}
XTEN288-Off7-MMAF	46.948	46.948	>95%	N.A.	N.A.	N.A.
XTEN576-Off7-MMAF	73.230	73.239	>95%	N.A.	N.A.	N.A.
XTEN864-Off7-MMAF	99.656	99.665	>95%	N.A.	N.A.	N.A.

[a] Molecular weights were calculated (MW_{calc}) with the ProtParam tool on the ExPASy server, based on the amino acid sequence of polypeptide-DARPin fusion constructs.

[b] Experimental molecular weights (MW_{exp_MS}) were determined by ESI-MS (cf. Fig. S6).

[c] Purity was determined by analytical hydrophobic interaction chromatography on an HPLC system (cf. Fig. S6). The purity is indicated as percentage and corresponds to the integrated conjugate peak area.

[d] Binding kinetics to EpCAM (hEpEx) were determined by surface plasmon resonance spectroscopy (SPR) (cf. Fig. S7).

Table S2: Cytotoxicity[a] of XTEN-DARPin-MMAF conjugates

Conjugate	**HT29 IC_{50}[b] (M)**	**SKBR3 IC_{50}[c] (M)**	**HEK 293T IC_{50}[d] (M)**
XTEN288-Ec1-MMAF	6.54×10^{-9}	2.82×10^{-9}	2.04×10^{-7}
XTEN576-Ec1-MMAF	7.91×10^{-9}	3.29×10^{-9}	2.08×10^{-7}
XTEN864-Ec1-MMAF	1.54×10^{-8}	4.75×10^{-9}	1.69×10^{-7}
XTEN288-Off7-MMAF	3.20×10^{-8}	8.79×10^{-8}	1.01×10^{-6}
XTEN576-Off7-MMAF	1.22×10^{-7}	2.28×10^{-7}	3.23×10^{-7}
XTEN864-Off7-MMAF	2.20×10^{-7}	1.20×10^{-7}	2.19×10^{-7}

[a] cytotoxicity was determined in XTT cell viability assays
(IC_{50}: concentration at which cell viability was decreased by 50%)

[b] HT29 is an EpCAM-positive cell line

[c] SKBR3 is an EpCAM-positive cell line

[d] HEK293T is an EpCAM-negative control cell line