Supporting Information

Active Targeting of Cancer Cells by Nanobody Decorated Polypeptide Micelle with Bioorthogonally Conjugated Drug

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Experimental Procedures

A. Materials

Cloning and protein expression: pET24+ vectors were purchased from Novagen (Madison, WI). Oligonucleotides and gBlocks encoding sequences of interest were purchased from Integrated DNA Technologies (Coralville, IA). Ligation enzymes, restriction enzymes, and calf intestinal alkaline phosphatase were purchased from New England Biolabs (Ipswich, MA). EB5α and BL21(DE3) chemically competent *E. coli* cells were purchased from Bioline (Taunton, MA). SHuffle T7 Express competent *E. coli* cells were purchased from New England Biolabs (Ipswich, MA). Genomically recoded *E. coli* cells (C321.ΔA) were generously provided by Prof. Farren Isaacs (Yale University). All *E. coli* cultures were grown in 2xYT media comprised of sodium chloride (5 g L^{-1}; Alfa Aesar, Ward Hill, MA), tryptone (16 g L^{-1}, Becton, Dickinson and Co., Franklin Lakes, NJ), and yeast extract (10 g L^{-1}, Becton, Dickinson and Co., Franklin Lakes, NJ). Kanamycin sulfate was purchased from EMD Millipore (Billerica, MA) and chloramphenicol was purchased from Sigma-Aldrich (St. Louis, MO). *p*-Acetylphenylalanine hydrochloride was purchased from Synchem, Inc. (Elk Grove Village, IL). Protein expression was induced with isopropyl β-d-1-thiogalactopyranoside (IPTG) from Gold Biotechnology (St. Louis, MO) and L-(+)-arabinose from Sigma-Aldrich (St. Louis, MO). 1× phosphate buffered saline (1xPBS) tablets (10×10^{-3} M phosphate buffer, 140×10^{-3} M NaCl, and 3×10^{-3} M KCl, pH 7.4 at 25°C) were purchased from EMD Millipore (Billerica, MA). Fluorescent dyes (AlexaFluor488-NHS ester, Alexa647 hydroxylamine) were purchased from Life Technologies (Grand Island, NY).

Doxorubicin conjugation: The linker for doxorubicin conjugation (O,O′-1,3-propanediylbishydroxylamine dihydrochloride) was purchased from Sigma-Aldrich (St. Louis, MO). Doxorubicin hydrochloride was purchased from CarboSynth (San Diego, CA). Aniline catalyst was purchased from Sigma-Aldrich (St. Louis, MO).

In vitro assays: Human cell lines A431, H69AR, HCT116, MDA-MB-468, SKOV-3, and OVCAR-3 were purchased from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. A431 cells were cultured in Dulbecco's Modified Essential Medium – High Glucose, supplemented with 10% fetal bovine serum (FBS). H69AR cells were cultured in RPMI-1640 supplemented with 20% FBS. HCT116 and SKOV-3 cells were cultured in McCoy's 5A Medium supplemented with 10% heat-inactivated FBS. MDA-MB-468 cells were cultured in Liebovitz's L-15 media supplemented with 10% FBS. OVCAR-3 cells were cultured in RPMI-1640 media supplemented with 0.01 mg/mL bovine insulin and 20% FBS. Transfected fibroblast lines were generously provided by Dr. Darell Bigner (Duke University) and cultured in Improved MEM Zinc Option (1X) (Gibco, Waltham, MA) supplemented with 10% heat-inactivated FBS. Cell culture media and FBS were purchased from Gibco (Waltham, MA). All cell culture media was supplemented with 1% penicillin/ streptomycin from Sigma (St. Louis, MO). With the exception of MDA-MB-468 cells, all cells were incubated at 37°C, 5% CO_2 atmosphere. MDA-MB-468 cells were incubated at 37°C with no supplemental CO_2. Cells were passaged every 2-3 days with 0.05% Trypsin/EDTA from Thermo Fisher (Hampton, MA). Cell viability assays were performed with CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) from Promega (Madison, WI).

B. Cloning

Genes encoding the ELP_{BC} and *p*AcF-ELP_{BC} were synthesized using recursive directional ligation by plasmid reconstruction, as described elsewhere.[1] The gene encoding the EgA1 nanobody was ordered as a gBlock (IDT Technologies, Skokie, IL) and introduced into the C-terminus of either ELP_{BC} or *p*AcF-ELP_{BC} by plasmid reconstruction, as described previously.[1]

We replaced the T7 promoter and terminator sequences of a modified pET-24+ vector with a pTac promoter and rrnB terminator, with vector re-construction performed by GenScript USA Inc. (Piscataway, NJ) as previously described.[2] This vector, m-pTac, is compatible with expression in the genomically recoded *E. coli* (C321.ΔA). We transferred the genes of interest by digesting the *p*AcF-ELP_{BC} and *p*AcF-ELP_{BC}-EgA1-containing m-pET-24+ vectors with BseRI/BamHI, extracting the insert using agarose gel separation and purification, and ligating with a similarly digested m-pTac. These vectors were then co-transformed with a modified pEvol tRNA/aaRS vector that contained two copies of the pAcFRS.1.t1 synthetase (generously provided by Prof. Farren Isaacs) into the C321.ΔA *E. coli* for expression with *p*AcF incorporation.

C. Protein expression

To express ELP_{BC}, liquid BL21DE3 *E. coli* cultures (50 mL) of strains harboring ELP plasmids (m-pET24+) were inoculated from frozen glycerol stocks and grown for 16-18h. Cultures were then inoculated at 1:20 dilution in 2xYT media (1 L) supplemented with kanamycin (45 μg ml^{-1}). Cells were grown at 37°C in a shaking incubator at 200 rpm for 6

h, at which time ELP expression was induced by the addition of IPTG (1 mM), and the cultures incubated at 37°C for an additional 18 h. Cell pellets were harvested by centrifugation at 3500xg and resuspended in 1xPBS (20 mL). Cells were lysed by sonication for a total of 3 min (Misonix; Farmingdale, NY) and DNA was precipitated by addition of polyethylenimine (10% w/v; MP Biomedicals, Santa Ana, CA). Precipitated DNA and cellular debris were removed by centrifugation at 20,000xg at 4 °C. Proteins were then purified using four rounds of inverse transition cycling, as described elsewhere[3]. Briefly, solutions of proteins were heated and salt (NaCl) was added to induce the phase transition of the ELP, centrifuged to collect all insoluble material at 35°C, 20,000xg ("hot spin"), and re-suspended in cold 1xPBS. Upon cooling, the ELP resolubilizes, while contaminants remain insoluble and can be removed by centrifugation at 4°C, 20,000xg ("cold spin").

To express *p*AcF-ELP$_{BC}$, liquid C321.ΔA *E. coli* cultures (50 mL) of strains harboring pEvol and ELP plasmids (m-pTac) were inoculated from frozen glycerol stocks and grown to confluence overnight. Cultures were then inoculated at 1:20 dilution in 2xYT media (1 L) supplemented with kanamycin (45 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}). aaRS expression was simultaneously induced by the addition of arabinose (0.2%) and *p*AcF (1 mM). Cells were grown at 34°C in a shaking incubator at 200 rpm for 6 h, at which time ELP expression was induced by the addition of IPTG (1 mM), and the cultures incubated at 34°C for an additional 18 h at which point cells were harvested and proteins purified as described above.

To express ELP$_{BC}$-EgA1, liquid SHuffle *E. coli* cultures (50 mL) of strains harboring the ELP plasmid (m-pET24+) were inoculated from frozen glycerol stocks and grown for 16-18h. Cultures were then inoculated at 1:20 dilution in 2xYT media (1 L) supplemented with kanamycin (45 μg ml^{-1}). Cells were grown at 30°C in a shaking incubator at 200 rpm for 6 h, at which time ELP expression was induced by the addition of IPTG (1 mm), and the cultures incubated at 16 °C for an additional 18 h. Cells were harvested, and proteins purified as described above.

To express *p*AcF-ELP$_{BC}$-EgA1, both C321.ΔA *E. coli* cultures harboring pEvol and the ELP plasmid (m-pTac) and SHuffle *E. coli* cultures harboring pEvol and the ELP plasmid (m-pET24+) were used as described above. Both expression hosts yielded proteins with *p*AcF incorporated (as determined by MALDI-TOF mass spectroscopy) and active EgA1 (as determined by flow cytometry), and *p*AcF-ELP$_{BC}$-EgA1 expressed from the C321.ΔA *E. coli* cultures were used for Dox conjugation studies.

Protein purity was characterized by 4–20% gradient Tris-HCl (Biorad, Hercules, CA) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with copper chloride (0.5 M; Thermo Fisher Scientific, Hampton, NH). Protein yield of *p*AcF-ELP$_{BC}$ was determined gravimetrically after dialysis into Nanopure water and lyophilization. Protein yield of nanobody-containing proteins (ELP$_{BC}$-EgA1, *p*AcF-ELP$_{BC}$-EgA1) was determined by UV/Vis spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA), using extinction coefficients of ε = 34505 cm^{-1} M^{-1} and ε = 37884.5 cm^{-1} M^{-1}, respectively.

D. Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed over a temperature range of 15−40°C using a Wyatt DynaPro temperature-controlled instrument (Wyatt Technology, Santa Barbara, CA). Samples for the DLS system were prepared in 1xPBS at a concentration of 25 μM and filtered through Whatman Anotop sterile syringe filters (0.2 μm; GE Healthcare Life Sciences, Pittsburgh, PA), into a quartz crystal cuvette (12 μL; Wyatt Technology, Santa Barbara, CA). Five acquisitions were taken at each temperature, and the results presented represent the mean R_h of the sample at each temperature. The data was analyzed with a regularization fit of the percent mass for Raleigh spheres. The error bars represent the polydispersity percentage determined from the five data points taken at each temperature.

E. Static light scattering

Static light scattering (SLS) measurements were performed using an ALV/CGS-3 goniometer system (Langen, Germany). Samples were prepared in 1xPBS and filtered through Whatman Anotop sterile syringe filters (0.02 μm) below the critical micellization temperature (CMT) into a disposable borosilicate glass tube (10 mm; Fischer Scientific, Pittsburgh, PA). After the sample was loaded into the laser path, the system equilibrated for 20 minutes at 35°C. Simultaneous SLS & DLS measurements were obtained at angles between 60°-150° at 5° increments, with each angle consisting of 3 runs for 15 s, at 35°C. The differential refractive index (dn dc^{-1}) was determined by measuring refractive index at six different concentrations at 35°C using an Abbemat 500 refractometer (Anton Paar, Graz, Austria). Static light scattering data were analyzed by partial Zimm plots using the ALVSTAT software to determine the radius of gyration (R_g) and molecular

weight. N_{agg} was determined by dividing the particle molecular weight by molecular weight of an individual pAcF-ELP_{BC} or *p*AcF-ELP_{BC}-EgA1 chain, as calculated from the amino acid sequence.

F. Cryogenic transmission electron microscopy (cryo-TEM)

Cryogenic transmission electron microscopy (cryo-TEM) imaging was performed at Duke University on an EI Tecnai G2 Twin TEM (FEI, Eindhoven, Netherlands) operated at 80 kV. Dox-*p*AcF-ELP_{BC} or Dox-*p*AcF-ELP_{BC}-EgA1 samples were adsorbed onto Lacey carbon grids (Ted Pella, Redding, CA). Lacey holey carbon grids (Ted Pella, Redding, CA) were glow discharged in a PELCO EasiGlow Cleaning System (Ted Pella, Redding, CA. Using unfiltered samples, a 3 μL drop (1 mg mL^{-1} of each construct) at 35°C (above the CMT) was deposited onto the grid, blotted for 3 s with a blot force of -3, and vitrified in liquid ethane at 100% relative humidity using the Vitrobot Mark III (FEI, Eindhoven, Netherlands). Grids were then transferred into a Gatan 626 cryoholder (GATAN Inc., Pleasanton, CA) and inserted into the TEM. 2D microscopic images were acquired using a FEI Eagle 4k × 4K CCD camera at varying magnifications.

G. Doxorubicin conjugation and purification

Doxorubicin was conjugated to *p*AcF-containing proteins via a two-step reaction: first, the telechelic linker was attached to the *p*-acetylphenylalanine (*p*AcF) residue via ketone condensation, and second, doxorubicin was attached to the linker via the same mechanism. After each reaction step, excess, unreacted products were removed. First, *p*AcF-ELP_{BC} or *p*AcF-ELP_{BC}-EgA1 were buffer exchanged from PBS into labeling buffer (50 mM sodium acetate, pH 5.0, 150 mM sodium chloride) and concentrated using by centrifugal ultrafiltration (Amicon Ultra-15, 10 kDa cut-off, Millipore Sigma, Burlington, MA) to 125 μM. The linker was resuspended in labeling buffer and twenty molar equivalents were added, along with aniline at a final concentration of 10 mM, which raised the pH of the reaction to pH 6.2. The reaction was carried out with stirring at 30°C in a mineral oil bath for 24 h. The linker-*p*AcF-ELP was then dialyzed against 8 L of PBS to remove excess linker and buffer exchanged into labeling buffer and concentrated to 125 μM by centrifugal ultrafiltration (Amicon Ultra-15). For the second reaction step, doxorubicin HCl was resuspended in water and ten molar equivalents added. Aniline was added to a final concentration of 10 mM. The second reaction was carried out with stirring in a mineral oil bath at 30°C for 24 hours. Excess doxorubicin was removed first with a PD-10 column (GE Healthcare, Chicago, IL) and then by washing with 15% acetonitrile/PBS by centrifugal ultrafiltration (Amicon Ultra-15). *p*AcF-ELP_{BC}-EgA1 concentration and reaction efficiency was determined by UV/Vis spectrophotometry (NanoDrop) with extinction coefficients $\varepsilon = 37{,}884.5\ cm^{-1}\ M^{-1}$ for *p*AcF-ELP_{BC}-EgA1 and $\varepsilon = 10{,}000\ cm^{-1}\ M^{-1}$ for Doxorubicin. The following formula (Eq. S1) was used to calculate the labeling of *p*AcF-ELP_{BC}-EgA1 with an A280 correction factor of 0.767:

$$\%\ \text{Labeling} = \frac{A_{490}/\varepsilon_{Dox}}{[A_{280}-(0.767\times A_{490})]/\varepsilon_{ELP}} \times 100\% \qquad \textbf{Eq. (S1)}$$

The concentration of *p*AcF-ELP_{BC} was determined gravimetrically by weighing the lyophilized protein and resuspending in a known volume of PBS.

H. Size exclusion chromatography (SEC)

For size exclusion chromatography analysis of the purity of Dox conjugates and fluorescently labeled proteins, a Shodex OHPak SB-804 column (New York, NY) was used with isocratic flow of 0.5 mL min^{-1} of PBS: acetonitrile [70:30] on a Shimadzu high performance liquid chromatography system (Shimadzu Scientific Instruments, Columbia, MD) . HPLC-grade solvents were purchased from VWR International (Radnor, PA).

I. pH release assay

To assay for the release of drug, samples of Dox-*p*AcF-ELP_{BC}-EgA1 (25 μM Dox equivalents) in PBS (pH 7.4) were buffer exchanged into either pH 4.0 (0.1 M sodium acetate) or pH 7.4 (PBS) buffers with centrifugal ultrafiltration (Amicon Ultra-0.5, 10 kDa MWCO). Samples were incubated at 37 °C for 3, 12, 24, 48, or 72 h and quenched by dilution 1:1 [vol:vol] into PBS, pH 7.4, to stop hydrolysis prior to analysis by HPLC. 100 μL of each sample at a 25 μM Dox equivalent concentration was analyzed using a LC10 HPLC (Shimadzu Scientific Instruments; Columbia, MD) on a Shodex OHPak KB-804 column (New York, NY) with isocratic flow of 0.5 mL min^{-1} of PBS: acetonitrile [70:30]. The integrated area under the curve (AUC) was quantified at an absorbance of 495 nm (A_{495}) corresponding to Dox. Two peaks eluted during the assay for Dox-*p*AcF-ELP_{BC}-EgA1 at 15.5 ± 0.5 min and for free Dox at 23.5 ± 0.5 min. The %

Dox released was determined by normalizing the AUC_{495} for the free Dox peak at each time point to the total AUC_{495} of the initial sample. Each condition was repeated for a total of n = 3 times.

J. Protein fluorescence labeling and flow cytometry

To fluorescently label proteins, 1 mg of AlexaFluor488-NHS ester was dissolved in 100 µL DMSO. The N-terminal amine of ELP_{BC} and ELP_{BC}-EgA1 were labeled with AlexaFluor488-NHS ester by incubating 100 µM protein with ten molar equivalents of dye, rotating for 24 h at 4°C in sodium phosphate buffer, pH 7.4. Excess unreacted dye was removed with a hot spin followed by washing with centrifugal ultrafiltration (Amicon Ultra-15, 10 kDa cut-off limit). Briefly, a hot spin consists of heating the solution of labeled protein above the T_t to initiate phase separation and centrifuging at high speeds to pellet the ELP, with free dye in the supernatant. The supernatant is removed, and the pellet is resuspended in cold PBS, whereupon the ELP resolubilizes. The purity of the labeled proteins was assessed by size exclusion chromatography. The following equations were used to calculate protein concentration [Eq. (S2)] and A488 concentration [Eq. (S3)].

$$[\text{AlexaFluor488}] = \frac{A_{494}}{73{,}000\ \text{cm}^{-1}\text{M}^{-1} \times 1\ \text{cm path length}} \qquad \textbf{Eq. (S2)}$$

$$[\text{ELP}_{BC}\text{-EgA1}] = \frac{A_{280} - (A_{494} \times 0.11)}{34{,}505\ \text{cm}^{-1}\text{M}^{-1} \times 1\ \text{cm path length}} \qquad \textbf{Eq. (S3)}$$

The concentration of ELP_{BC} and labeling efficiency was determined gravimetrically after dialysis into DI water, lyophilization, weighing, and resuspension in 1xPBS. The labeling efficiency of both proteins by Alexa488 was ~25%.

Overnight cultures of adherent cells were prepared for flow cytometry by first trypsinizing cells with 0.05% trypsin/EDTA and harvesting the cells by centrifugation for 3 min at 1000xg. The cell pellet was resuspended in PBS/1% BSA, the cells were counted with a hemocytometer after 1:1 dilution into Trypan blue solution (0.4%, Thermo Fisher Scientific, Waltham, MA), and the cell density adjusted to 2×10^6 cells mL^{-1}. Cells were incubated with fluorescently labeled proteins by gently mixing 90 µL of the cell suspension with 10 µL of 100 µM ELP (25 µM Alexa488) followed by a 1 h incubation with rocking at 4°C to minimize protein uptake by the cells. Flow experiments with incubation at 37°C ensure micelle self-assembly showed equivalent or higher levels of cell binding as those performed at 4°C (data not shown). After incubation, cells were collected and washed 3x with 0.5 mL PBS/1% BSA on ice, with a final cell concentration prior to flow analysis of 2×10^5 cells mL^{-1}. Live cells were analyzed for population fluorescence on a BD FACSCanto Analyzer (BD Biosciences, San Jose, CA) at the Duke Cancer Institute Flow Cytometry Shared Resource Facility to determine the geometric mean fluorescence intensity (gMFI) of samples.

K. Tryptic digests, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and electron spray ionization liquid chromatography/mass spectrometry (ESI-LC/MS)

*p*AcF-containing samples were designed such that the leader sequence contains a trypsin-cleavable arginine residue following the stop codon to allow for mass spectrometry analysis of *p*AcF incorporation. Lyophilized trypsin was reconstituted using 50 mM acetic acid to 1 mg mL^{-1} as per the manufacturer's instructions. Samples of *p*AcF-ELP_{BC} and *p*AcF-ELP_{BC}-EgA1 at 100 µM were incubated with MS-grade trypsin protease (Pierce, Waltham, MA) at a ratio of 20:1 in 50 mM ammonium bicarbonate, pH 8. These reactions were incubated for 16 hours at 37°C and then analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a Bruker Autoflex Speed LRF MALDI-TOF System. Digested samples were mixed at a ratio of 1:1 with α-cyano-4-hydroxycinnamic acid (HCCA) matrix and 2 µL deposited onto a ground steel target plate and dried in air at room temperature. All spectra were calibrated against adrenocorticotropic hormone fragment 18-39 (Sigma Aldrich, St. Louis, MO).

For ESI-LC/MS, tryptic digests were similarly prepared and analyzed with an Agilent 1100 Series LC/MSD Trap SL (Agilent Technologies, Santa Clara, CA). Samples were injected into a Phenomenex Luna C18 column (50 x 1 mm, 3 µm; 0.2% formic acid in water as buffer A, 0.2% formic acid in acetonitrile as buffer B) and then into the mass spectrometer using a fully automated system. Spectra were acquired in positive mode followed by analysis and deconvolution using LC/MSD Trap Data Analysis software (Agilent Technologies, Santa Clara, CA). Mass spectra were acquired at the Mass Spectrometry Shared Facility at Duke University. Incorporation and reaction step efficiencies was calculated by taking the ratio of intensities for the peak of a specific product over the total peak intensities of all relevant peaks in the sample.

L. Cytotoxicity assay

The in vitro cytotoxicity of Dox conjugates was determined by a colorimetric assay, as follows. 0.5×10^3 A431 or SKOV-3 cells were seeded per 40 µL complete media on BD Falcon™ 384-well cell culture plates (BD; Franklin Lakes, NJ) and allowed to adhere for 16–18 h. After adherence, 10 µL of serial dilutions of Dox, Dox-*p*AcF-ELP_{BC}, or Dox-*p*AcF-ELP_{BC}-EgA1 was added to the wells and incubated at 37°C for 24 h. After the treatment period, 10 µL of CellTiter 96 AQueous™ (Promega; Madison, WI) 3-(4,5,-dimethyl2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium (MTS) reagent was added to each well. Following incubation for 3 hours, the absorbance of the solution was measured twice at 490 nm and 650 nm with a Victor3 microplate reader (Perkin Elmer; Waltham, MA). The background A650 was subtracted from the A490 readings to determine the cell viability [Eq. (S5)] as compared to untreated controls according to the following equation. Wells containing media only with equivalent concentrations of Dox were also prepared to subtract background contribution from free Dox.

$$\%\ \text{Viability} = \frac{\text{Corr. A490} - \text{A490}_{\text{Media}}}{\text{Corr. A490}_{\text{Untreated}} - \text{A490}_{\text{Media, Untreated}}} \times 100\% \qquad \textbf{Eq. (S5)}$$

To calculate the IC_{50}, the data was fit to a sigmoidal curve and used in the following equation [Eq. (S6)]:

$$\%\ \text{Viability} = \frac{1}{1 + \left(\frac{C_{Dox}}{IC_{50}}\right)^{p}} \qquad \textbf{Eq. (S6)}$$

where C_{Dox} is the effective Dox concentration in the well, the IC_{50} measures the necessary dose to kill 50% of the cell population, and *p* represents the slope of the sigmoidal curve.

M. Fluorescent micelle uptake

For fluorescence visualization of ELP_{BC}-EgA1 uptake, 4×10^4 transfected fibroblast cells were seeded on Lab-Tek® II CC2™ chamber slides (Electron Microscopy Sciences, Hatfield, PA) and allowed to adhere for 18 h. Cell media was replaced with complete media containing either (1) 10 µM AlexaFluor488-ELP_{BC}-EgA1, (2) 10 µM AlexaFluor488-ELP_{BC}, or (3) 10 µM AlexaFluor488-ELP_{BC}-EgA1 with 100 µM excess unlabeled ELP_{BC}-EgA1, and incubated for 24 h at 37 °C. Following treatment, the media was removed, and cells were incubated for 10 min with 2 µM Hoechst 33342 (Life Technologies, Carlsbad, CA) to stain cell nuclei and 5 µg mL^{-1} wheat germ agglutinin (WGA) AlexaFluor 594 (Thermo Fisher Scientific, Waltham, MA) to stain cell membranes. The cells were then washed twice with Hank's Balanced salt solution (HBSS, Thermo Fisher Scientific, Waltham, MA) at room temperature. The slide was mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA) prior to visualization on a Nikon TE-2000U widefield fluorescence microscope with a 60× oil-immersion objective. Hoechst 33342 dye was detected with a standard UV-2E/C filter set, WGA was detected with a 540/25 nm excitation filter, 565 nm long pass dichromatic mirror, and 605-655 nm band pass emission filter set, and Dox was detected with a 450–490 nm excitation filter, 505 nm long pass dichromatic mirror, and 590–650 nm emission filter set.

N. Confocal microscopy for EGF competition and colocalization experiments

To confirm specificity of EgA1 nanobody for EGF, 1.5×10^4 transfected fibroblasts were seeded in 110 µL of complete media into each of the four chambers of a µ-Dish$^{35\ mm,\ high}$, Culture-Insert 4 Well (1.5 coverslip, Ibidi, Madison, WI) and allowed to adhere for 18 h. Cell media was replaced with complete media containing either 2 µg mL^{-1} pHrodo Green EGF (Invitrogen, Carlsbad, CA) or fresh media and incubated for 1 h at 37°C. pHrodo Green EGF is a weakly fluorescent, pH-sensitive dye that is brightly fluorescent only after endocytosis into cells via EGFR. Cells were then washed twice with Hank's Balanced salt solution (HBSS, Thermo Fisher Scientific, Waltham, MA) and complete cell media containing 10 µM Alexa647-ELP_{BC}-EgA1 was added to the wells and cells incubated for 1 h at 37°C. Following this incubation, the media was gently removed and replaced with complete media containing 2 µM Hoechst 33342 (Life Technologies, Carlsbad, CA) to stain cell nuclei for 10 min at 37°C. Cells were then washed twice with HBSS at room temperature and maintained in complete media prior to imaging on an Andor Dragonfly Spinning Disk 500 series confocal on a LeicaDMi8 microscope stand (Oxford Instruments, Abingdon, UK) with a 63× water immersion objective and equipped with a Zyla 4.2 series camera. Hoechst 33342 dye was detected with a 400 nm excitation filter and 450/50 nm emission filter, pHrodo Green EGF with a 488 nm excitation laser and 525/50 nm emission filter, and Alexa647-ELP_{BC}-EgA1 with a 561 nm excitation laser and 600/50 nm emission filter in CF40 imaging mode.

For colocalization of Dox conjugates with lysosomal compartments, 2.75×10^4 A431 cells were seeded in 110 μL of complete media into each of the four chambers of a μ-Dish$^{35\ mm,\ high}$, Culture-Insert 4 Well (1.5 coverslip, Ibidi, Madison, WI) and allowed to adhere for 18 h. Cell media was replaced with complete media containing either 25 μM Dox-*p*AcF-ELP_{BC}-EgA1 or 25 μM Dox-*p*AcF-ELP_{BC} and incubated for either 4 or 24 h at 37°C. After treatment, the media was gently removed and replaced with complete media containing 1x CytoPainter LysoDeep Red Indicator Reagent (Abcam, Cambridge, MA) to stain lysosomal compartments and incubated for 30 min at 37°C. Following this incubation, the media was gently removed and replaced with complete media containing 2 μM Hoechst 33342 (Life Technologies, Carlsbad, CA) to stain cell nuclei for 10 min at 37°C. Cells were then washed twice with Hank's Balanced salt solution (HBSS, Thermo Fisher Scientific, Waltham, MA) at room temperature and maintained in fresh HBSS prior to imaging on an Andor Dragonfly Spinning Disk 500 series confocal on a LeicaDMi8 microscope stand (Oxford Instruments, Abingdon, UK) with a 63× water immersion objective and equipped with a Zyla 4.2 series camera. Hoechst 33342 dye was detected with a 400 nm excitation filter and 450/50 nm emission filter, Dox with a 488 nm excitation laser and 525/50 nm emission filter, and CytoPainter with a 637 nm excitation laser and 700/75 nm emission filter in CF40 imaging mode. Imaging processing and colocalization analysis was performed using the Coloc2 plug-in available with FIJI (ImageJ, National Institutes of Health).

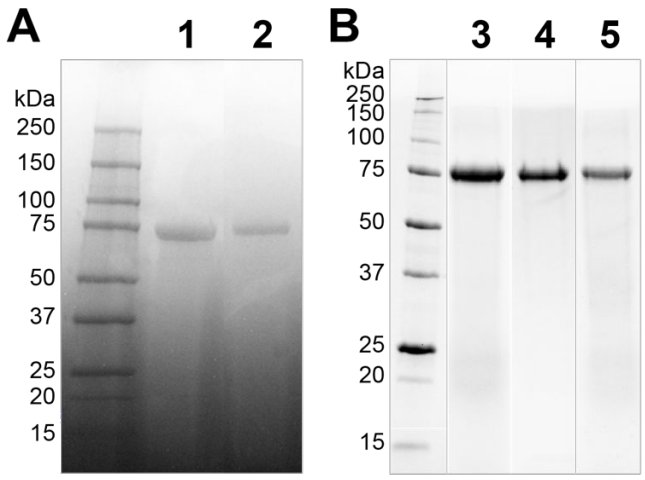

Figure S1. SDS-PAGE of all constructs expressed in this study. (A) Bands visualized with copper chloride stain. Lane 1: ELP_{BC}, 64.8 kDa. Lane 2: *p*AcF-ELP_{BC}, 66.0 kDa. (B) Tryptophan-containing proteins visualized on stain-free gel. Lane 3: ELP_{BC}-EgA1, 72.5 kDa. Lane 4: *p*AcF-ELP_{BC}-EgA1, 73.9 kDa. Lane 5: Dox-*p*AcF-ELP_{BC}-EgA1, 74.4 kDa. ELPs and their fusions migrate anomalously on SDS-PAGE because of their biased amino acid distribution compared to the globular proteins in the MW standards.

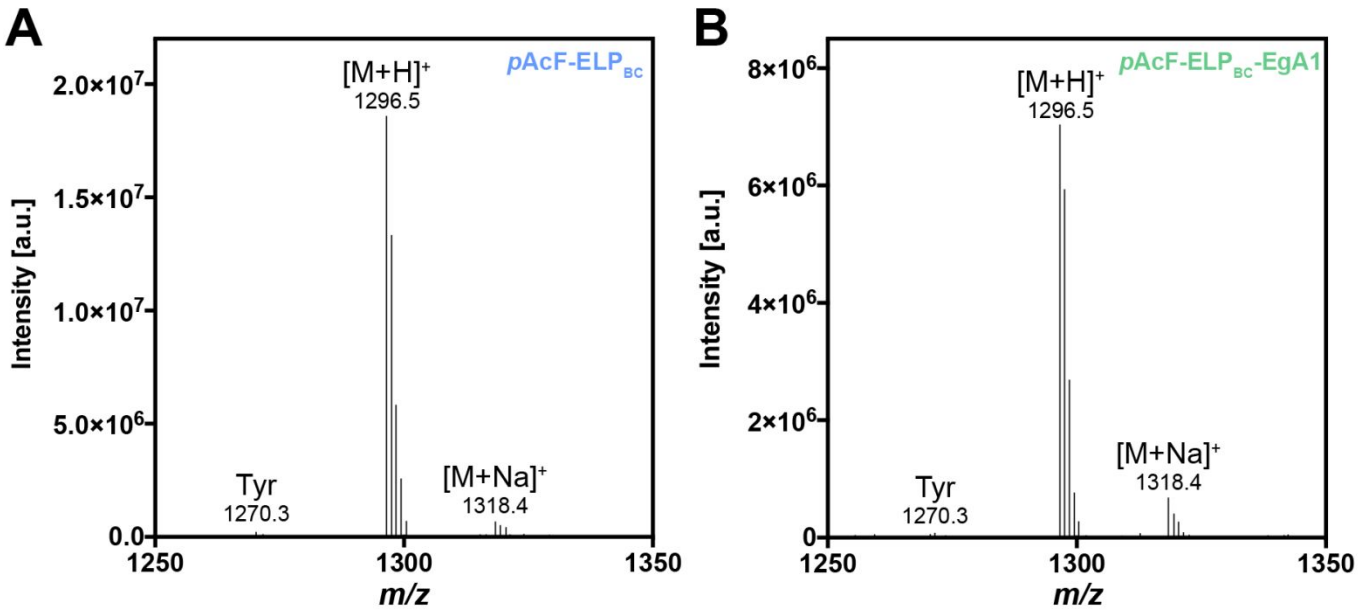

Figure S2. Confirming *p*AcF incorporation into *p*AcF-ELP$_{BC}$ and *p*AcF-ELP$_{BC}$-EgA1 with ESI-LC/MS. Tryptic digests of (A) *p*AcF-ELP$_{BC}$ and (B) *p*AcF-ELP$_{BC}$-EgA1 were analyzed to quantify extent of *p*AcF incorporation as well as identify any misincorporation products with natural residues. Expected molecular weight of *p*AcF-containing peptide is 1296.66 Da. Peptides with Tyr, Trp, and Phe were detected as minor populations, with Tyr representing the primary residue misincorporated in both samples.

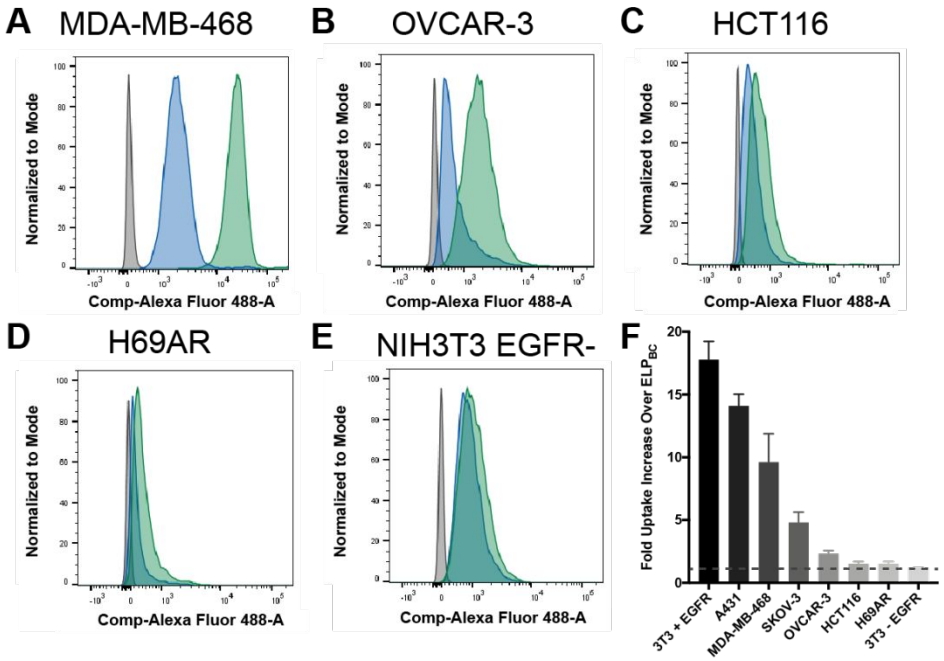

Figure S3. Flow cytometry of fluorescently labeled ELP_{BC}-EgA1 (green populations) and ELP_{BC} (blue populations) in (A) MDA-MB-468 mammary carcinoma; (B) OVCAR-3 ovarian carcinoma; (C) HCT116 colorectal carcinoma; (D) H69AR small cell lung cancer; and (E) untransfected murine fibroblasts that do not express human EGFR. (F) Quantified fold uptake increase of ELP_{BC}-EgA1 over ELP_{BC} demonstrate the range of EGFR expression across the panel of cell lines tested as well as lack of non-specific ELP_{BC}-EgA1 uptake in the untransfected fibroblasts. Dotted line indicates equivalent uptake as ELP_{BC} (fold uptake = 1).

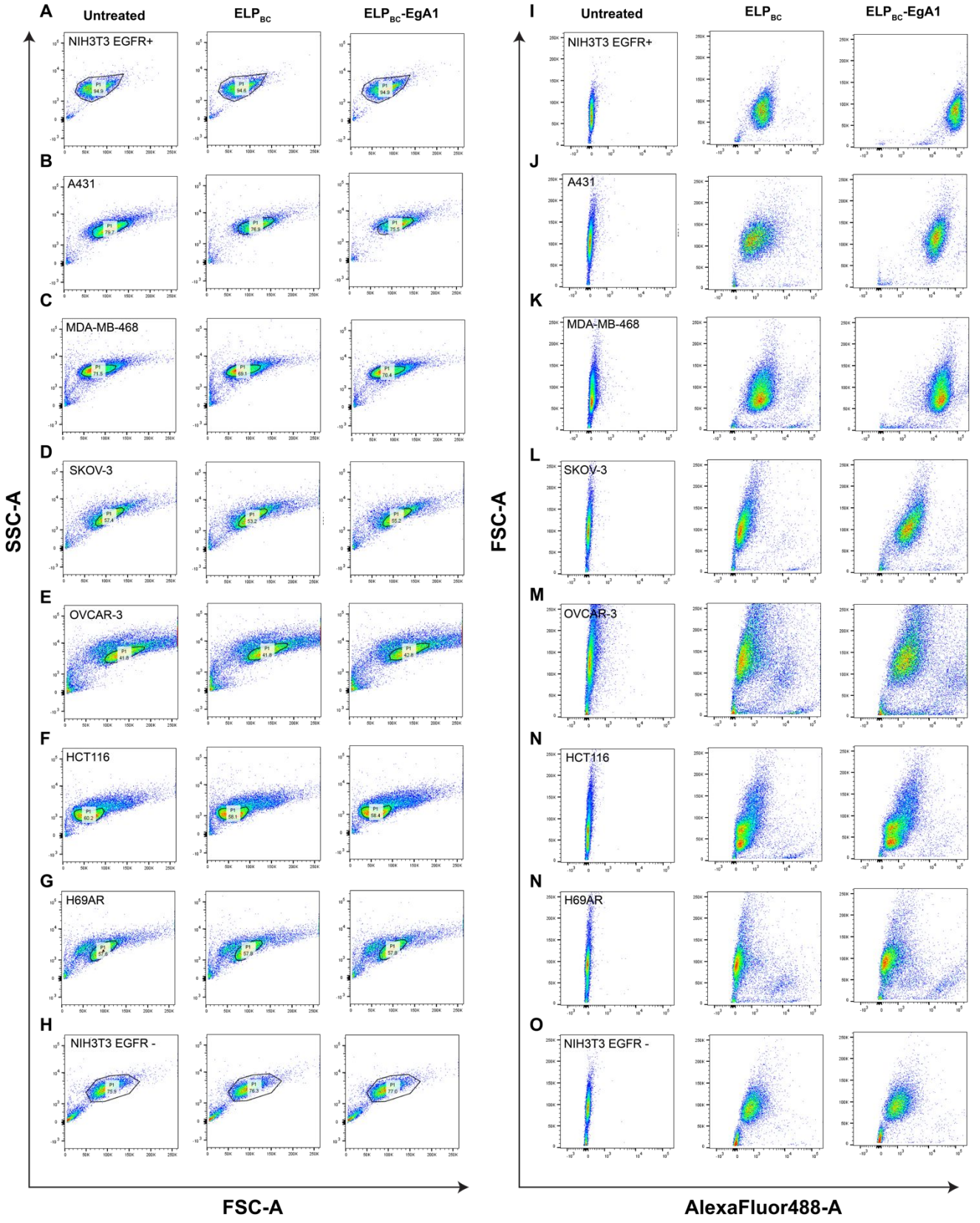

Figure S4. Scatterplots of flow cytometry data for panel of cell lines tested. (A-H) SSC-A versus FSC-A and population gating used. (I-O) FSC-A versus AlexaFluor488-A.

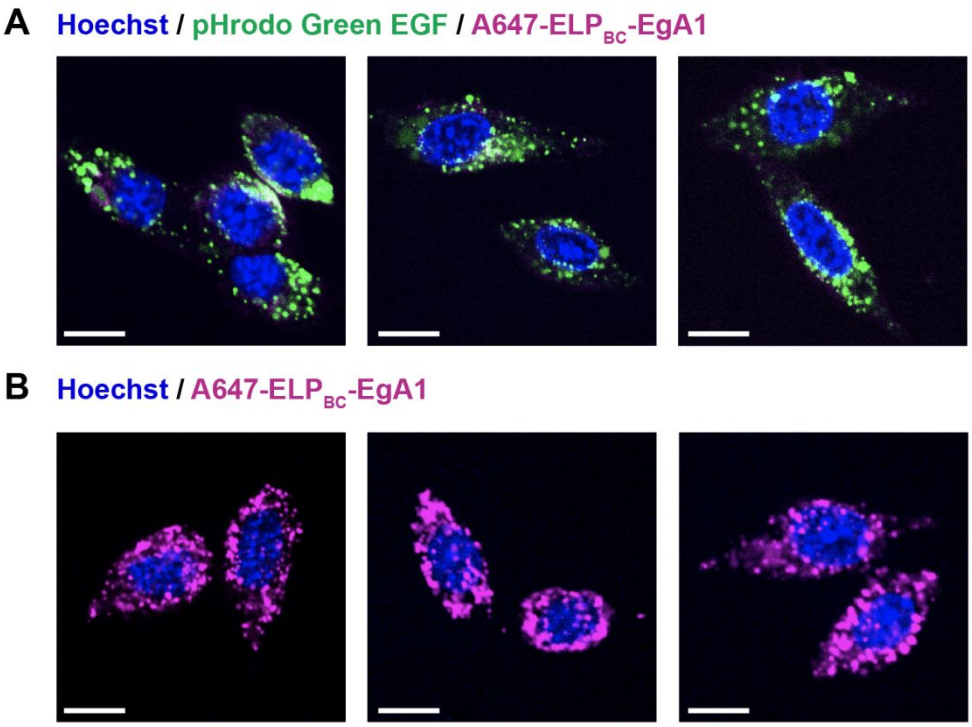

Figure S5. Confirming EGFR specificity of EgA1 nanobody by EGF competitive uptake assay. NIH 3T3 murine fibroblasts transfected with human EGFR were (A) pre-incubated with excess fluorescently labeled pHrodo Green EGF, followed by AlexaFluor647-ELP$_{BC}$-EgA1 or (B) treated with AlexaFluor647-ELP$_{BC}$-EgA1 alone. Hoechst stains cell nuclei in both panels. Scale bars 20 μm.

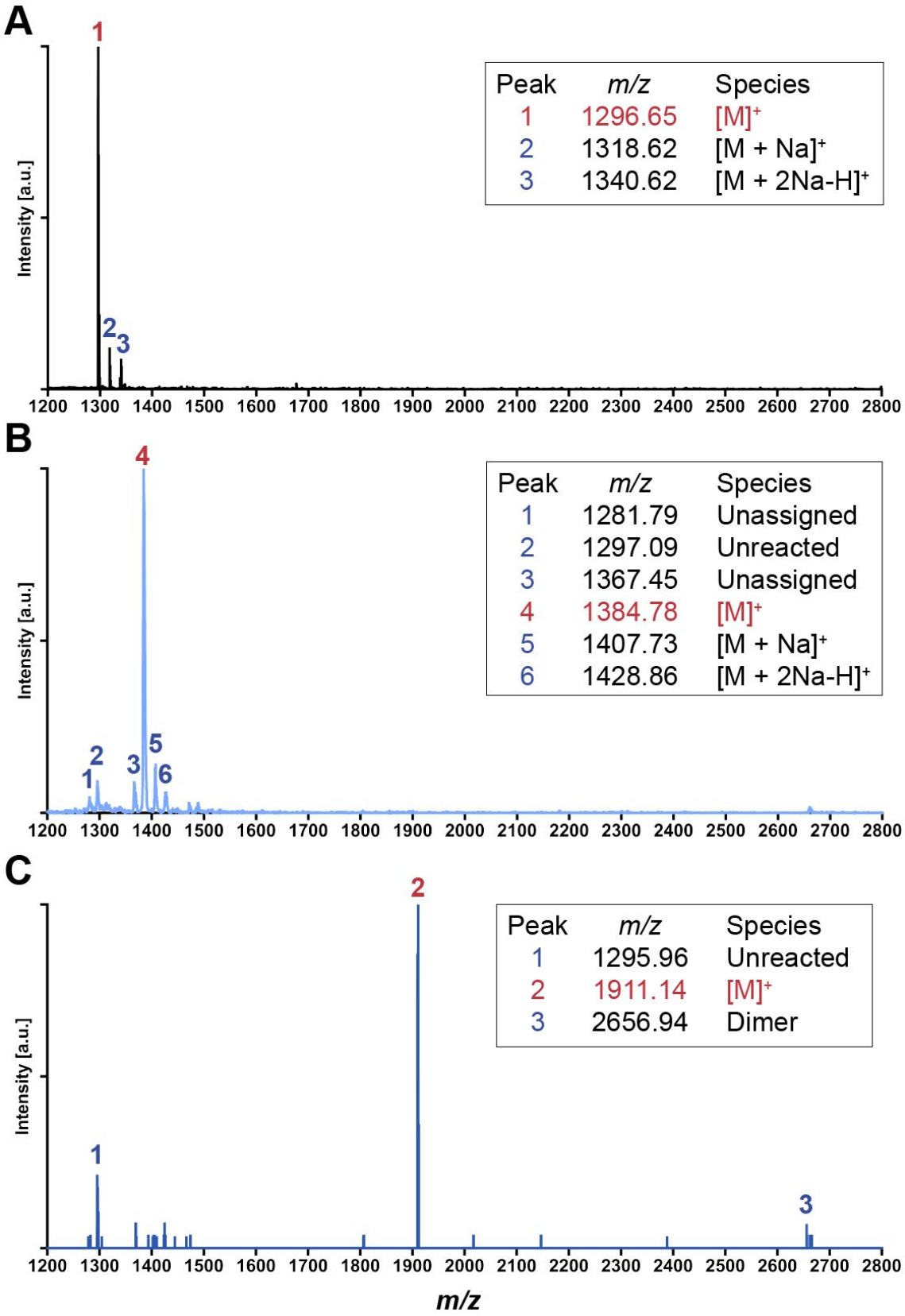

Figure S6. Complete MALDI-TOF-MS spectra of Dox reaction steps after tryptic digest. (A) Starting material, *p*AcF-ELP$_{BC}$. (B) Intermediate reaction step after addition of linker shows a minor population of unreacted starting material. (C) Final reaction product Dox-*p*AcF-ELP$_{BC}$ shows minor populations of unreacted starting material and dimer between *p*AcF-containing peptides.

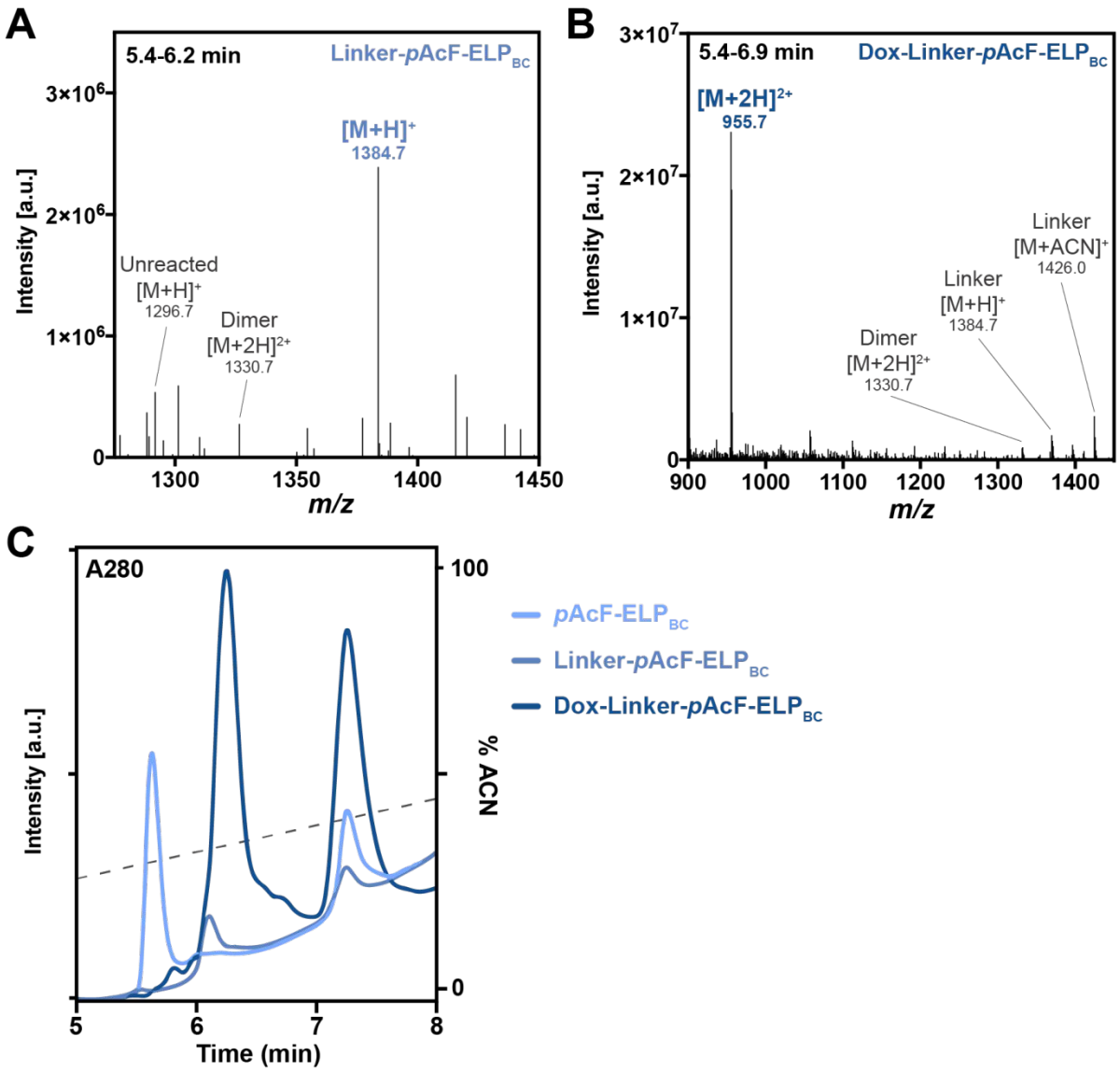

Figure S7. Evaluating efficiency of Dox reaction steps with ESI-LC/MS. Tryptic digests of (A) linker-*p*AcF-ELP$_{BC}$ and (B) Dox-linker-*p*AcF-ELP$_{BC}$ were analyzed to quantify reaction efficiency and identify the composition of the reaction mixture after each reaction step. (C) A280 UV chromatogram of starting material and samples from each reaction step show two peaks: the first peak (elution time 5 - 7 min) corresponds to the digested peptide with retention time increasing with reaction progress. The second peak (elution time 7 - 8 min) corresponds to the remainder of the protein resulting from tryptic digest.

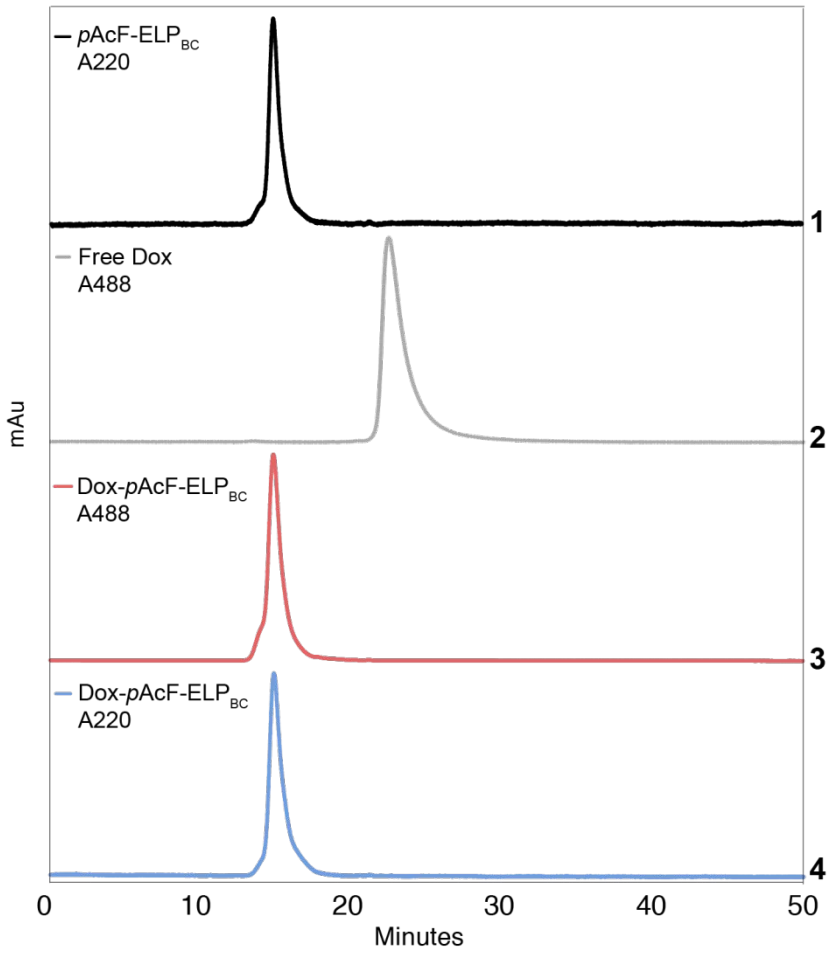

Figure S8. Confirmation of Dox conjugation to Dox-*p*AcF-ELP$_{BC}$ using size exclusion chromatography (SEC). *p*AcF-ELP$_{BC}$ elution at 15.5 min (trace 1, black) is detected by its absorbance at 220 nm. Free Dox is detecting by its absorbance at 488 nm and elutes at 23.5 min (trace 2, grey). In the Dox-*p*AcF-ELP$_{BC}$ conjugates, (traces 3 and 4) no free Dox is observed and the Dox absorbance at 488 nm (trace 3, red) co-elutes with that of ELP, which absorbs at 220 nm (trace 4, blue). In addition to confirming conjugation, traces also demonstrate the purity of the conjugate with no free Dox peak.

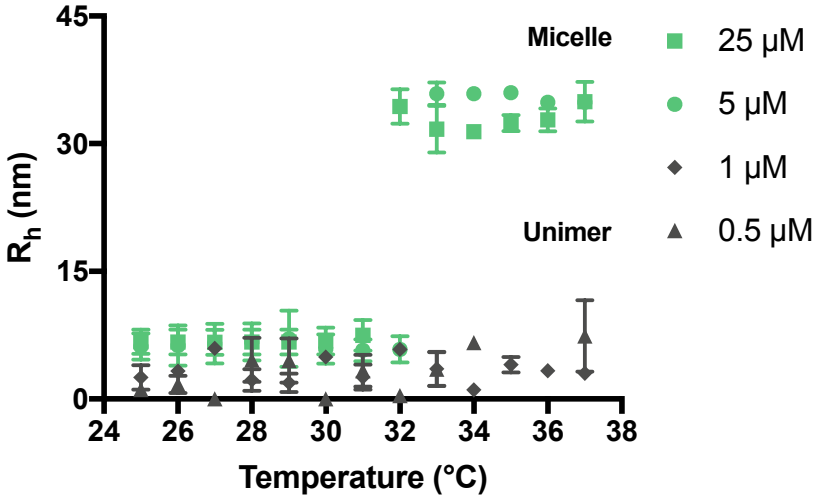

Figure S9. Approximating critical micellization concentration (CMC) of Dox-*p*AcF-ELP_{BC}-EgA1 by analyzing dilution series with dynamic light scattering (DLS). Two samples with concentrations above the CMC (green; 25 μM and 5 μM) self-assemble into micelles above the critical micellization temperature (CMT), approximately 32°C. In contrast, the samples with concentrations below the CMC (grey; 1 μM and 0.5 μM) remain as unimers throughout the temperature ramp.

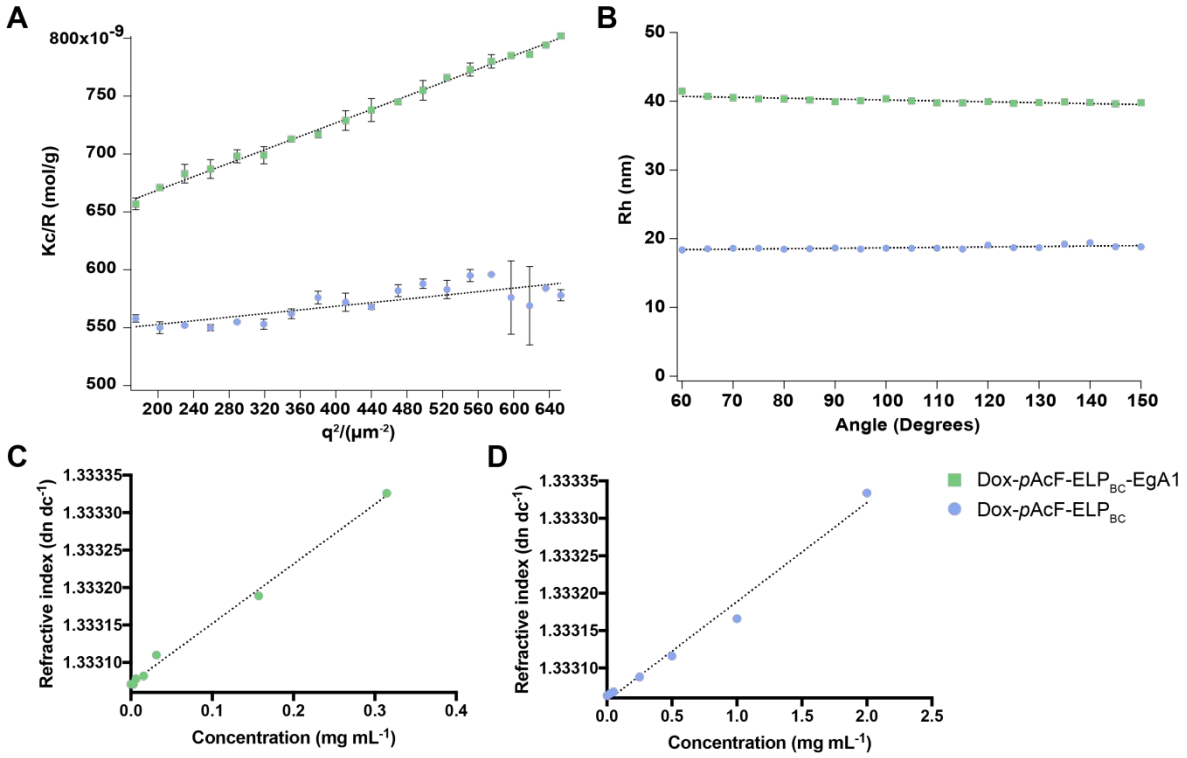

Figure S10. Static light scattering characterization of Dox conjugates. (A) Partial Zimm plot of Dox-*p*AcF-ELP$_{BC}$-EgA1 (green) and Dox-*p*AcF-ELP$_{BC}$ (blue) with dashed lines showing best linear fit and error bars represent the standard deviation (n = 5 measurements). (B) Dynamic light scattering analysis of hydrodynamic radius as a function of scattering angle of Dox-*p*AcF-ELP$_{BC}$-EgA1 (green) and Dox-*p*AcF-ELP$_{BC}$ (blue). Refractive index of (C) Dox-*p*AcF-ELP$_{BC}$-EgA1 and (D) Dox-*p*AcF-ELP$_{BC}$ as a function of concentration. All data was collected at 35°C.

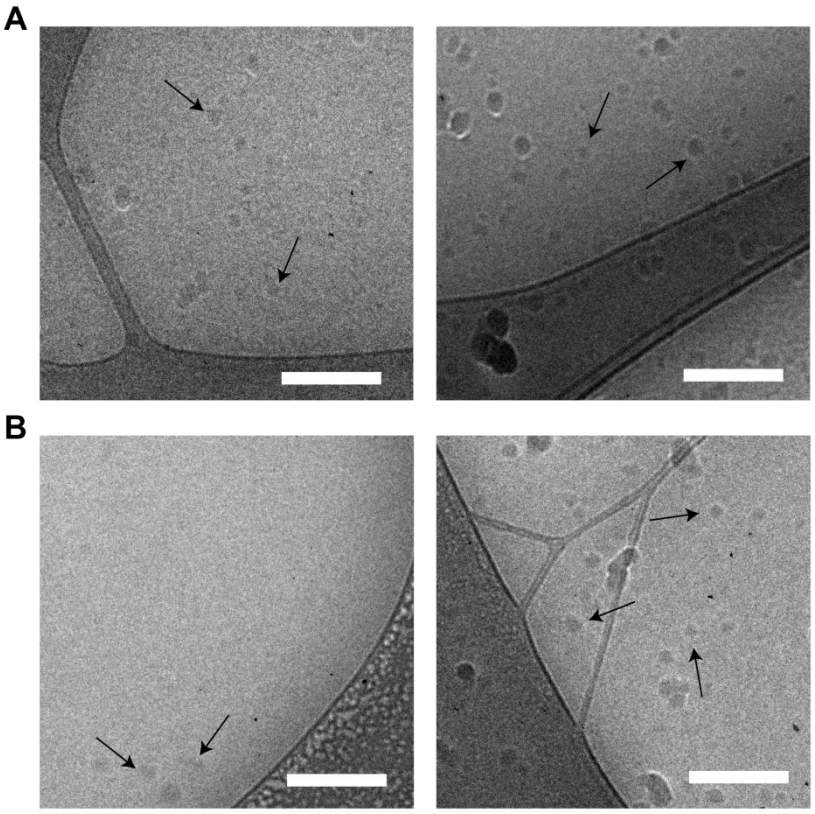

Figure S11. Additional Cryo-TEM images of (A) Dox-*p*AcF-ELP$_{BC}$-EgA1 and (B) Dox-*p*AcF-ELP$_{BC}$. Scale bar, 200 nm.

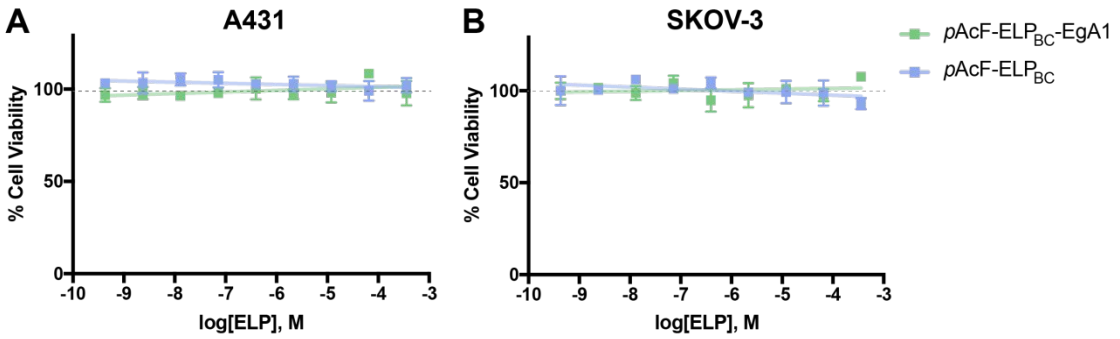

Figure S12. Control cell viability assays demonstrate that *p*AcF-ELP_{BC} (blue) and *p*AcF-ELP_{BC}-EgA1 (green) are not cytotoxic in (A) A431 and (B) SKOV-3 cells. The concentrations of ELP assayed correspond to the concentrations of these constructs in the Dox conjugates tested.

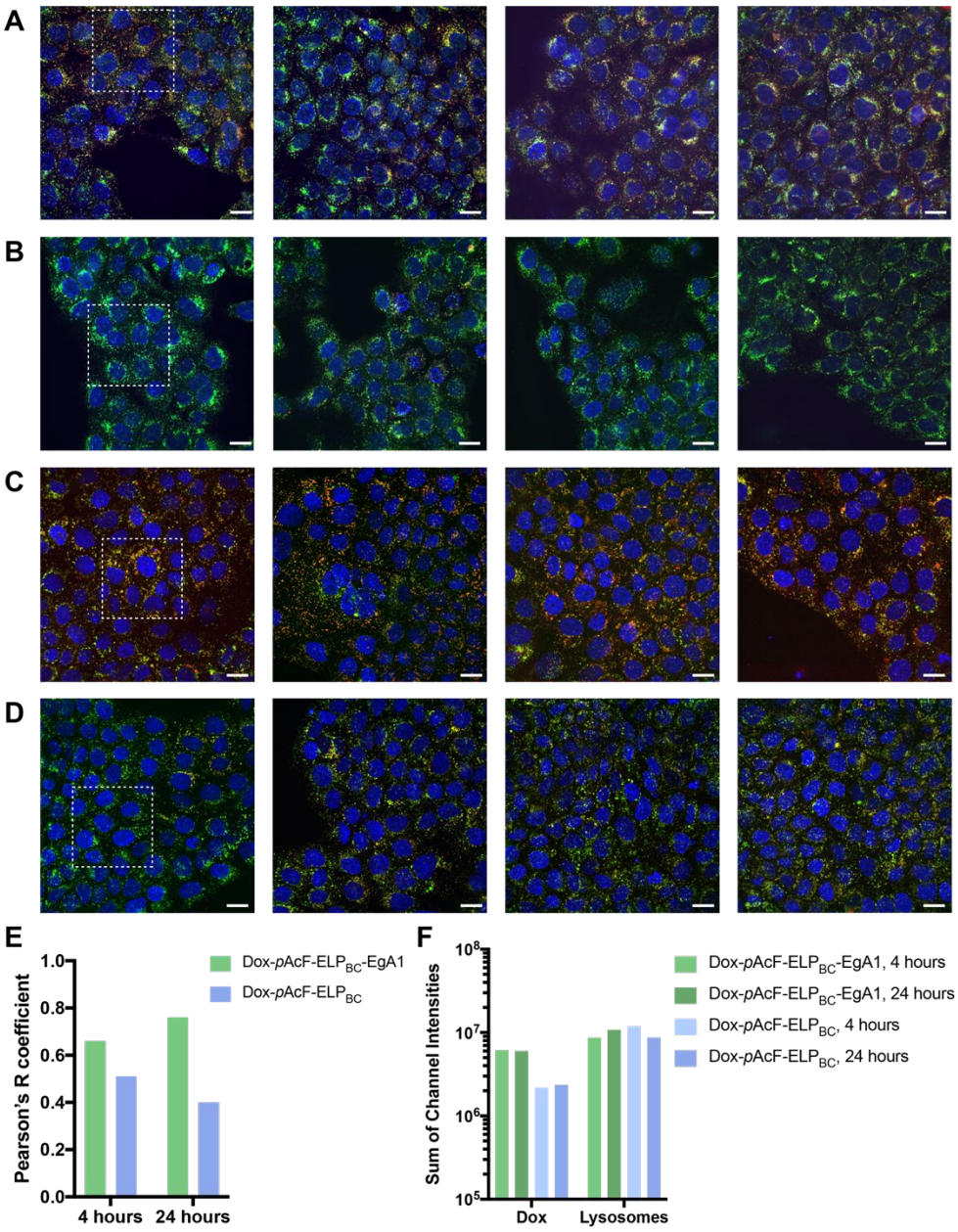

Figure S13. Additional confocal microscopy images of A431 cells incubated with Dox constructs to show wider field of view. Areas designated by dashed white squares indicate regions shown and analysed in Figure 5. (A) Dox-*p*AcF-ELP$_{BC}$-EgA1, 4 h incubation; (B) Dox-*p*AcF-ELP$_{BC}$, 4 h incubation; (C) Dox-*p*AcF-ELP$_{BC}$-EgA1, 24 h incubation; (D) Dox-*p*AcF-ELP$_{BC}$, 24 h incubation. Scale bars 20 μm. (E) Pearson's R coefficient for colocalization of areas analysed in Figure 5 show higher levels of colocalization between Dox and lysosomes for Dox-*p*AcF-ELP$_{BC}$-EgA1 at both 4 and 24 h times points as compared to Dox-*p*AcF-ELP$_{BC}$. (F) Sum of channel intensities for Dox and lysosomes for images analysed for colocalization to ensure lysosomal channel intensity is similar between images analysed and does not account for the differences in colocalization observed.

Table S1. Amino acid sequences and molecular weights of the constructs used in this study.

Name	Sequence[a]	MW (Da)
ELP_{BC}	GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGV PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG VGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGY	64791.60
*p*AcF-ELP_{BC}	**GVGVPG*p*AcFGVPGVGR**GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGV PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG VGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGY	66088.33
ELP_{BC}-EgA1	GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGV PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG VGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVP GSGVPG**EPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAASGRTFSSYAMGWFRQAPG** **KQREFVAAIRWSGGYTYYTDSVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCAATYLSS** **DYSRYALPQRPLDYDYWGQGTQVTVSSLE**	72582.54
*p*AcF-ELP_{BC}-EgA1	**GVGVPG*p*AcFGVPGVGR**GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGV PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG VGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGSGVPGS GVPGSGVPGSGVPGSGVPGSGVPGSGVPG**EPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAASG** **RTFSSYAMGWFRQAPGKQREFVAAIRWSGGYTYYTDSVKGRFTISRDNAKTTVYLQMNSL** **KPEDTAVYYCAATYLSSDYSRYALPQRPLDYDYWGQGTQVTVSSLE**	73879.02

[a] Blue text shows *p*AcF-containing leader sequence; orange text shows flexible hinge separating ELP from EgA1 nanobody, derived from the long hinge region of the *Llama glama* IgG3 heavy chain antibody;[4] EgA1 nanobody sequence in green text.

Table S2. Summary of *E. coli* strains, growth conditions, and yield of constructs in this study.

Construct	*E. coli* line	Temperature	Yield (mg L^{-1})
ELP_{BC}	BL21(DE3)	37°C	160
*p*AcF-ELP_{BC}	C321.ΔA	34°C	30
ELP_{BC}-EgA1	SHuffle T7 Express	30°C / 16°C	50
*p*AcF-ELP_{BC}-EgA1	C321.ΔA	34°C	15
	SHuffle T7 Express	30°C / 16°C	12

Table S3. Percent composition of Dox reaction products

Construct, reaction step	Component	% Composition
*p*AcF-ELP$_{BC}$, starting material	*p*AcF	98.72
	Tyr	1.17
	Trp	0.11
*p*AcF-ELP$_{BC}$-EgA1, starting material	*p*AcF	98.37
	Tyr	0.81
	Phe	0.63
	Trp	0.16
Linker- *p*AcF-ELP$_{BC}$, reaction step 1	Linker-*p*AcF-ELP$_{BC}$	85.10
	*p*AcF-ELP$_{BC}$	5.03
	ELP$_{BC}$-*p*AcF-linker-*p*AcF-ELP$_{BC}$ dimer	9.87
Dox-Linker- *p*AcF-ELP$_{BC}$, reaction step 2	Dox-linker-*p*AcF-ELP$_{BC}$	93.45
	Linker-*p*AcF-ELP$_{BC}$	1.33
	*p*AcF-ELP$_{BC}$	0.83
	ELP$_{BC}$-*p*AcF-linker-*p*AcF-ELP$_{BC}$ dimer	4.37

Table S4. Reaction conditions and labelling efficiency of Dox reactions

Reaction	Temperature	pH	Catalyst	Dox Labeling (%)[a]
*p*AcF-ELP$_{BC}$	30°C	4.0	--	74.5 ± 1.5
*p*AcF-ELP$_{BC}$	30°C	6.2	10 mM aniline	90.9 ± 5.6
*p*AcF-ELP$_{BC}$-EgA1	30°C	6.2	10 mM aniline	80.7 ± 6.1

[a] n ≥ 3; variability reported as standard error of the mean

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