Supporting Information for Bellucci, et al, "A non-canonical function of sortase enables site-specific conjugation of small molecules to a lysine residue in proteins"

SUPPLEMENTARY FIGURE CAPTIONS

Supp. Fig. 1. Overview of the transpeptidation reaction catalyzed by SrtA in *S. aureus*. (a) As proteins bearing the LPXTG sortase recognition site are secreted through the cell membrane, SrtA breaks the peptide bond between the threonine and glycine residues and forms an enzyme-substrate intermediate through a thioester bond between the threonine residue and the catalytic cysteine. (b) The acyl-enzyme intermediate is stable but is resolved after nucleophilic attack by the α-amino group of a pentaglycine branch of the cell wall. (c) After the transpeptidation reaction is complete, the catalytic thiol of SrtA is regenerated and the substrate protein is anchored to the peptidoglycan through a native peptide bond.

Supp. Fig. 2. Schematic of pilin assembly by sortases in Gram-positive bacteria. (a) Secreted pilin monomer protein (SpaA) is cleaved by a pilin-specific sortase, forming a thioester-linked intermediate. (b) The pilin polymer extends after the intermediate undergoes nucleophilic attack by the ε-amino group of a lysine in the next monomer, forming an isopeptide bond. (c) After a stochastic number of extension steps, a housekeeping sortase terminates polymerization by anchoring the assembled pilin chain to a pentaglycine branch in the peptidoglycan through a native peptide bond.

Supp. Fig. 3. Thermal responsiveness of the SrtA-ELP fusion protein used in this study. (a) Absorbance at 350 nm was measured for 100, 75, 50, and 25 μM SrtA-ELP concentrations as the temperature was ramped from 15°C-35°C at 1 ºC/min in sortase reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 8.5). The transition temperature is defined as the inflection point of the absorbance as a function of temperature. (b) Plotting the transition temperature versus SrtA-ELP concentration allows prediction of the SrtA-ELP transition temperature at a particular concentration by interpolation or extrapolation. Turbidity profiles for protein-ELP fusions were characterized using a Cary 300 Bio UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) and concentrations were determined by the Beer-Lambert Law using calculated extinction coefficients and the absorbance at 280 nm measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Supp. Fig. 4. Reaction with peptide substrates demonstrates the isopeptide ligation reaction catalyzed by sortase A. (a) Scheme showing the sortase-mediated isopeptide ligation of a peptide containing the pilin domain with a peptide containing the sortase recognition sequence LPETG and an amino-terminal biotin. (b) MALDI-TOF mass spectrometry indicates the formation of a reaction product with a molecular weight corresponding to peptides linked through an isopeptide bond when

SrtA-ELP is included in the reaction (upper panel), but not when the enzyme is omitted from the reaction mixture (lower panel).

Supp. Fig. 5. Expected peptides from tryptic digestion of the unpurified product of the SrtA-ELP-catalyzed biotinylation of Fn3-PLN$_3$-ELP. The table shows the tryptic peptide sequences for peaks identified in the MALDI-TOF spectra in Figures 2c, 2d, and Supp. Fig. 5. Included are the predicted molecular weight, the experimentally measured molecular weight (*m/z* value – H^+ mass), and the difference between these two values. The complete sequences of SrtA-ELP and Fn3-PLN$_3$-ELP are provided for reference. Excellent agreement between the predicted and measured peptide molecular weights was observed for all ions in the MALDI-TOF spectra. Biotinylation was specific to the pilin domain lysine, as peptides corresponding to biotinylation at the α-amino group of Fn3-PLN$_3$-ELP or through the ε-amino groups of any of the 3 lysines in Fn3 were not found in the MALDI-TOF spectrum. The structure of biotin-LPET is also provided for reference.

Supp. Fig. 6. (a) Schematic of the SrtA-ELP-catalyzed reaction of biotin-LPETGRAGG peptide and Fn3-PLN$_3$-ELP, showing all peptides that would be expected for different sites of biotin conjugation. (b)-(c) Control reactions for the MALDI-TOF spectrum shown in Figures 2c and 2d. (b) MALDI-TOF spectrum for biotinylation reaction containing Fn3-ELP (no pilin domains) and biotin-LPETGRAGG peptide did not contain any ions corresponding to biotinylated peptides (this spectrum corresponds to lane 1 of the Western blot in Figure 2b). (c) MALDI-TOF spectrum for reaction of Fn3-ELP (no pilin domains) without biotin-LPETGRAGG peptide did not contain ions corresponding to biotinylated peptides (this spectrum corresponds to lane 3 of the Western blot in Figure 2b). As expected, these spectra do not contain ions corresponding to biotinylated pilin domains (ions **1** and **2**), as they are not present in the substrate protein.

Supp. Fig. 7. His-tagged SrtA achieves site-specific conjugation of biotin to the pilin domain lysine. (a) SDS-PAGE of reactions of H$_6$-SrtA and Fn3-PLN$_3$-ELP run overnight at a range of temperatures from 21-42°C with or without biotin-LPETGRAGG peptide. (b) Western blot of the gel in panel (a) using streptavidin-Cy5 to detect biotinylated protein. Only reactions containing both the pilin domain and the biotin-LPETGRAGG peptide result in biotinylation of the target protein at 43 kDa. H$_6$-SrtA appears as a biotinylated band at ~25 kDa in reactions containing biotin-LPETGRAGG because the thioester intermediate of H$_6$-SrtA and biotin-LPET is stable in SDS-PAGE. A disulfide-linked dimer of the enzyme also appears as a biotinylated protein in the blot due to the amino-terminal glycine of the enzyme, which can be biotinylated by the native peptide transpeptidation reaction at its α-amino group. (c) The MALDI-TOF mass spectrum of tryptic peptides of the unpurified 20°C biotinylation reaction contains ions that correspond to the same biotinylated pilin domain peptides (ions

1 and **2**) as in Figure 2a, as well as a peptide corresponding to the amino-terminus of the H_6-SrtA enzyme biotinylated through a native peptide bond at the terminal glycine residue.

Supp. Fig. 8. FITC can be conjugated to substrate proteins by sortase-mediated isopeptide ligation. (a) FITC-Ahx-LPETGRAGG peptide was reacted with Fn3-PLN_3-ELP and SrtA-ELP overnight at 32°C in reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$). (b) The reaction product was analyzed by SDS-PAGE and total protein was visualized (upper panel). Scanning the gel with a Typhoon scanner with 488 nm laser excitation and a 520 BP 40 nm emission filter showed that Fn3-PLN_3-ELP was conjugated to FITC (lower panel). SrtA-ELP is also visible in this image because the thioester reaction intermediate of SrtA and the FITC-LPET peptide is stable in SDS-PAGE. (c) The reaction product was trypsinized and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. The MALDI-TOF spectrum showed the presence of two ions with *m/z* 3040.7 and 4148.7, which correspond to the predicted molecular weight of pilin domain tryptic peptides linked to FITC-Ahx-LPET through an isopeptide bond at the pilin domain lysine. As with the biotin conjugation reactions, these peptides contain a missed tryptic digestion at the pilin domain lysine because trypsin is unable to cleave at these lysines after isopeptide bond formation. (d) The chemical structure of the FITC-Ahx-LPET moiety attached to the pilin domain lysine.

Supp. Fig. 9. The biotinylation reaction of Fn3-PLN_3-ELP was purified with cation exchange chromatography (CEX). (a) Absorbance at 280 nm, pH, and conductivity traces for the CEX run. The biotinylation reaction product was loaded on a HiTrap SP FF 5 ml column at 5 ml/min, washed in 20 mM Tris, pH 6.0, and eluted in a gradient to 100% 1M NaCl, 20 mM Tris, pH 6.0 over 50 minutes. Non-bound (NB) and elution (Elu) fractions were collected. (b) SDS-PAGE of the CEX load, non-bound, and elution fractions indicated that Fn3-PLN_3-ELP was selectively recovered in the elution fraction. (c) A Western blot of the CEX load, non-bound (NB), and elution (Elu) fractions using streptavidin-Cy5 indicates successful recovery of biotinylated Fn3-PLN_3-ELP in the elution.

Supp. Fig. 10. Fully annotated version of the MS1 spectrum for LC-MS/MS corresponding to Figure 3a and Supp. Fig. 9. A peak table with the ions shown in the spectra is also shown.

Supp. Fig. 11. (a) MS2 spectrum of daughter ions produced by isolating the +3 charge state (*m/z* = 920.4) of a biotinylated pilin domain peptide. The spectrum shows four ions with multiple charge states whose masses confirm the expected chemical structures for biotin-LPET linked to the pilin domain lysine through an isopeptide bond (ions **1**-**4**). These fragments

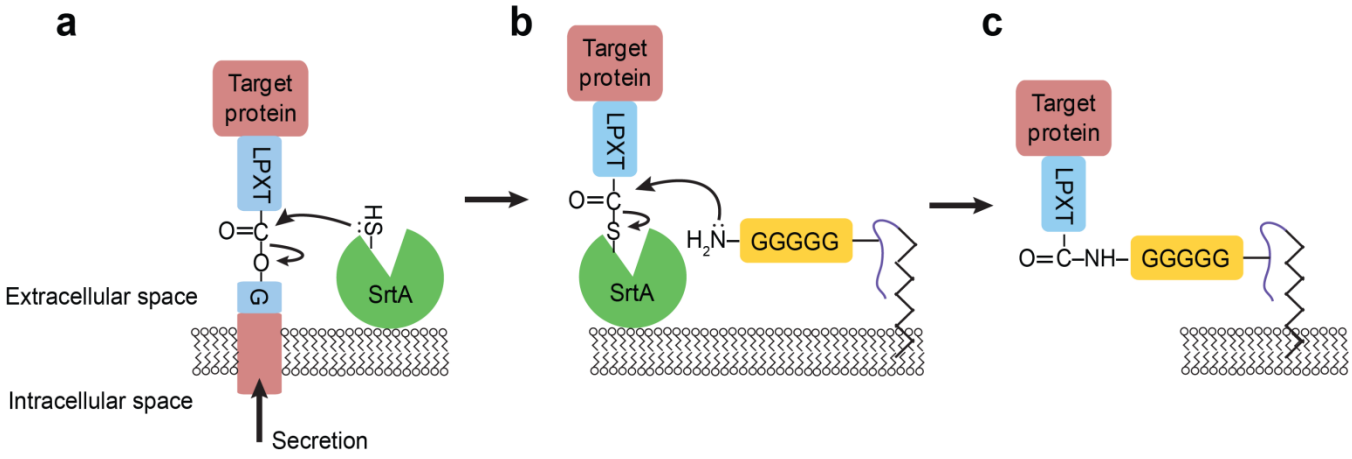
correspond to y- and b-type ions produced by breaking peptide bonds along the pilin domain backbone (ions **1**, **3**, and **4**) as well as within the LPET linker region (ions **1** and **2**). (b) Outline of the observed fragmentation pattern and the nomenclature used to classify daughter ions, with colors corresponding to the daughter ion chemical structures in panel (c). Peptide bond cleavage results in a charged amino-terminal fragment (b ions) or a charged carboxy-terminal fragment (y ions). b_{IP} and y_{IP} correspond to daughter ions from fragmentation events within the isopeptide-linked LPET moiety.

Supp. Fig. 12. Additional images for SK-OV-3 immunofluorescence. (a-c) Cells were stained with a 1:200 dilution of anti-Her2 (with a pilin domain on each heavy chain) biotinylated by reaction with sortase. (d-f) Cells were stained with a 1:200 dilution of biotinylated murine IgG1 isotype control antibody. Biotin in both groups was detected by secondary staining with streptavidin-FITC conjugate (green). Nuclei were stained with Hoechst 33342 (blue). Scale bars are 15 μm.

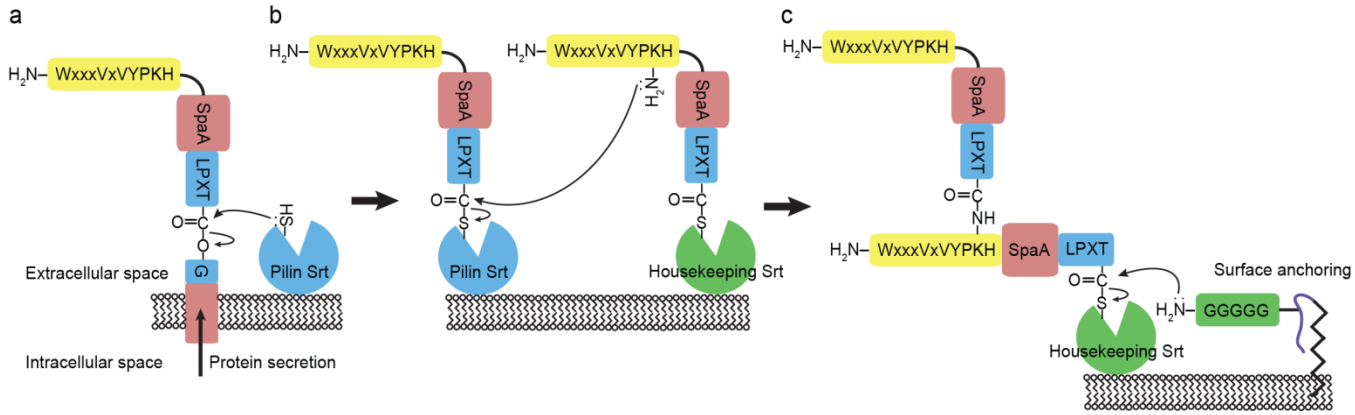
Supp. Fig. 13. Quantitation of the biotin-to-protein molar ratio for anti-Her2 with one pilin domain inserted at the carboxy-terminus of each heavy chain. Antibody was biotinylated by overnight reaction at 32°C with SrtA-ELP and biotin-LPETGRAGG peptide in 2- and 100-fold molar excess respectively. Purified antibody was dialyzed extensively (30 diavolumes) with PBS to remove unreacted biotin. Protein-bound biotin was quantified using a fluorescence biotin detection kit with a known concentration of biocytin as a standard, according to the manufacturer's instructions as described in the Supplementary Methods section. Protein concentration was determined by BCA assay using a bovine serum albumin standard per the manufacturer's instructions. Biotin and protein content were evaluated for undiluted antibody as well as 4 dilutions of the conjugate (1:4, 1:6, 1:8, and 1:10). Plotting the concentration of biotin versus the concentration of protein for each sample gives a slope of 1.8 with a standard error of 0.1, which indicates that 90% of the pilin domains in the antibody sample were biotinylated in the isopeptide ligation reaction. All samples and standards were measured in triplicate and error bars indicate standard deviations.

SUPPLEMENTARY FIGURES

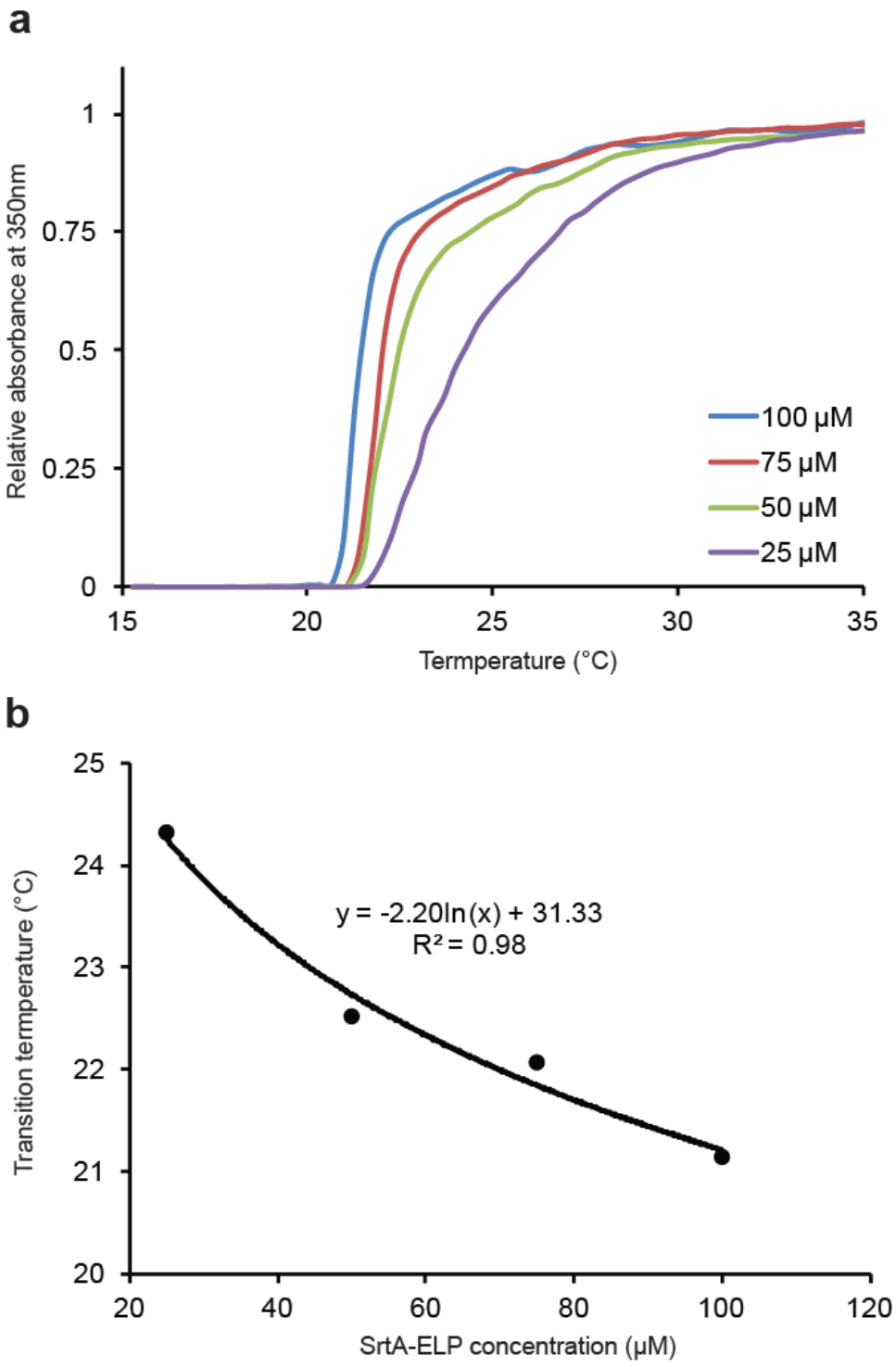
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



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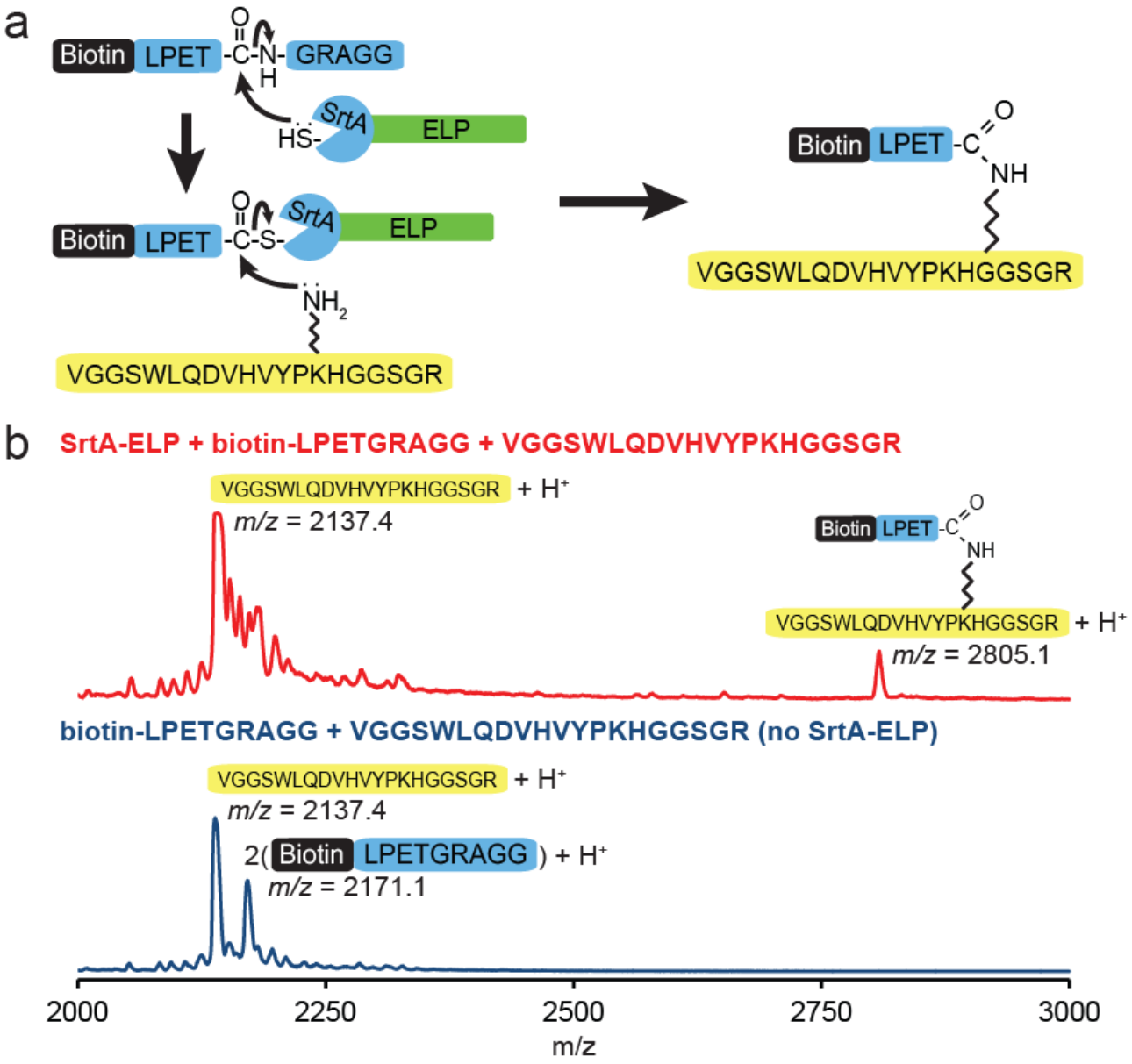


Supplementary Figure 5.

SrtA-ELP amino acid sequence

MGQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATPEQLNRGVSFAEENESLDDQNISIAGHTFIDRPNYQFTNLKA
AKKGSMVYFKVGNETRKYKMTSIRDVKPTDVGVLDEQKGKDKQLTLITCDDYNEKTGVWEKRKIFVATEVKALV
TMGVG-$(VPGVG)_{240}$

Fn3-PLN_3-ELP amino acid sequence

MVSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVT
PRGDWNEGSKPISINYRTGGTSGGTSGSGSGGGSGGWLQDVHVYPKHGGSGRGSGGWLQDVHVYPKHGGSGRGS
GGWLQDVHVYPKHGGSGRGVGV-$(VPGVG)_{60}$

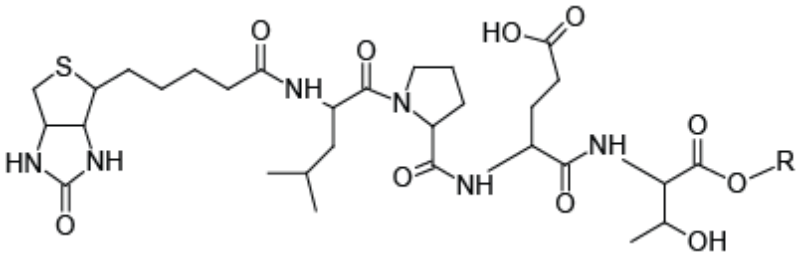
Fn3-ELP amino acid sequence

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTPR
GDWNEGSKPISINYRTGGTSGGTSGSGSGGGVG-$(VPGVG)_{60}$

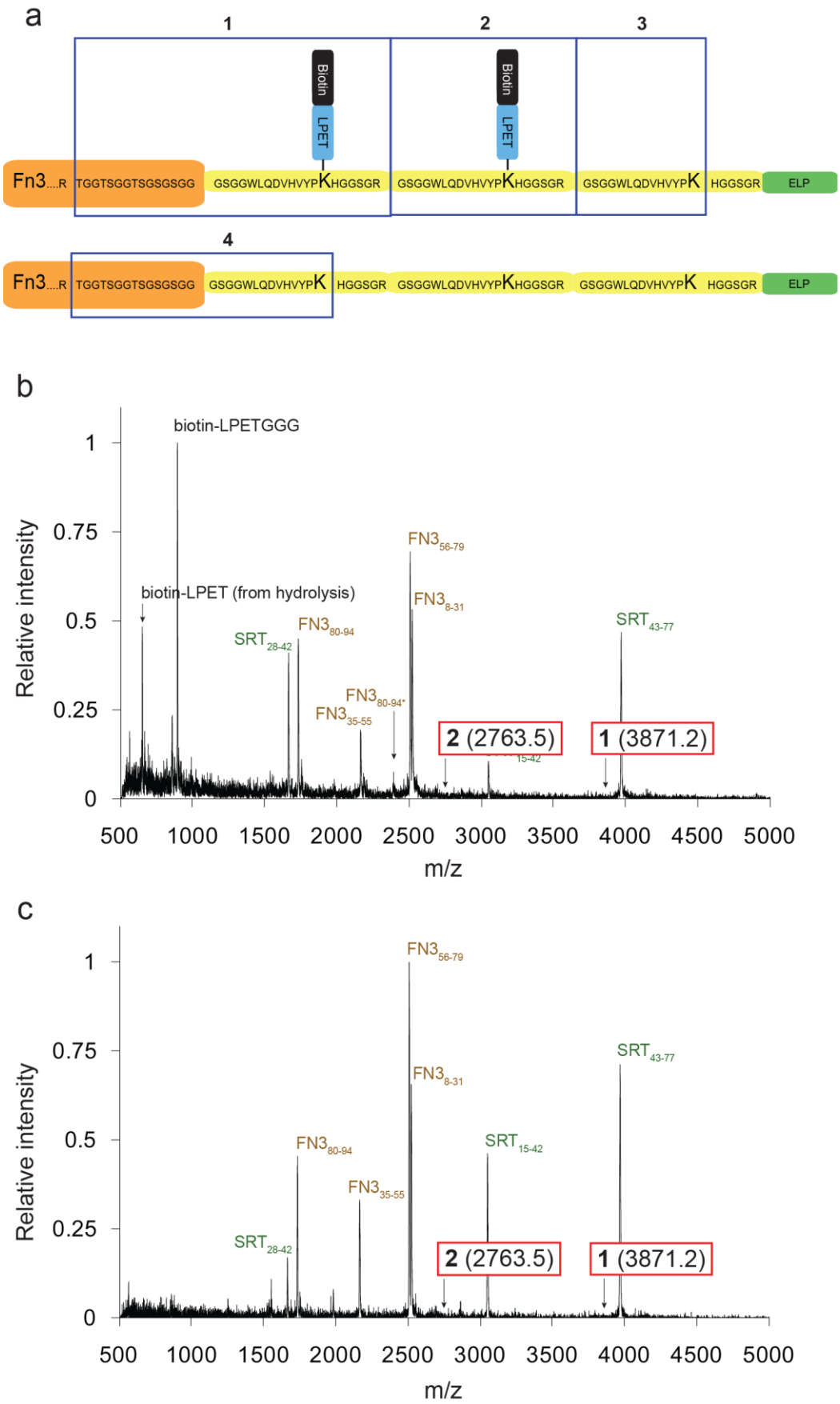
Peak table for tryptic peptides of SrtA-ELP reaction with Fn3-PLN_3-ELP and biotin-LPETG

Peak Label	Peptide sequence	Predicted mass (Da)	Found mass [m/z - H^+] (Da)	Difference (Da)
1	TGGTSGGTSGSGSGGGSGGWLQDVHVYPKHGGSGR + biotin-LPET	3203.3 + 666.8 = 3870.1	3870.2	-0.1
2	GSGGWLQDVHVYPKHGGSGR + biotin-LPET (attachment to lysine ε-amino group)	2095.3 + 666.8 = 2762.1	2762.5	-0.4
3	GSGGWLQDVHVYPK	1543.7	1543.5	0.2
4	TGGTSGGTSGSGSGGGSGGWLQDVHVYPK	2651.8	2651.6	0.2
Biotin-LPETGGG	Biotin-LPETGGG + $2Na^+$	666.8 + 189.2 + 44 = 900.0	900.6	-0.6
SRT_{28-42}	EPVYPGPATPEQLNR	1668.9	1669.0	-0.1
SRT_{15-42}	VAGYIEIPDADIKEPVYPGPATPEQLNR	3054.4	3053.5	0.9
$SRT_{121-149}$	QLTLITCDDYNEKTGVWEKRKIFVATEVK	3430.0	3430.8	-0.8
SRT_{43-77}	GVSFAEENESLDDQNISIAGHTFIDRPNYQFTNLK	3972.3	3972.3	0.0
$Fn3_{80-94}$	GDWNEGSKPISINYR	1736.9	1736.2	0.7
$Fn3_{35-55}$	ITYGETGGNSPVQEFTVPGSK	2169.4	2168.4	1.0
$Fn3_{80-94*}$	Biotin-LPET + GDWNEGSKPISINYR (through α-amino group)	666.8 + 1736.9 = 2403.7	2403.7	0.0
$Fn3_{56-79}$	STATISGLKPGVDYTITVYAVTPR	2511.9	2512.1	-0.2
$Fn3_{8-31}$	DLEVVAATPTSLLISWDAPAVTVR	2525.9	2526.2	-0.3
	Biotin-LPET + MVSDVPR (attachment to amino-terminus through α-amino group)	666.8 + 804.0 = 1470.8	Not found	N/A
	Biotin-LPET + VSDVPR (attachment to amino-terminus through α-amino group, methionine loss)	666.8 + 672.8 = 1339.6	Not found	N/A
	STATISGLKPGVDYTITVYAVTPR + biotin-LPET (attachment to lysine ε-amino group in Fn3)	2511.9 + 666.8 = 3178.7	Not found	N/A
	ITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTPR + biotin-LPET (attachment to lysine ε-amino group in Fn3)	4662.2 + 666.8 = 5329.0	Not found	N/A

Structure of Biotin-LPET



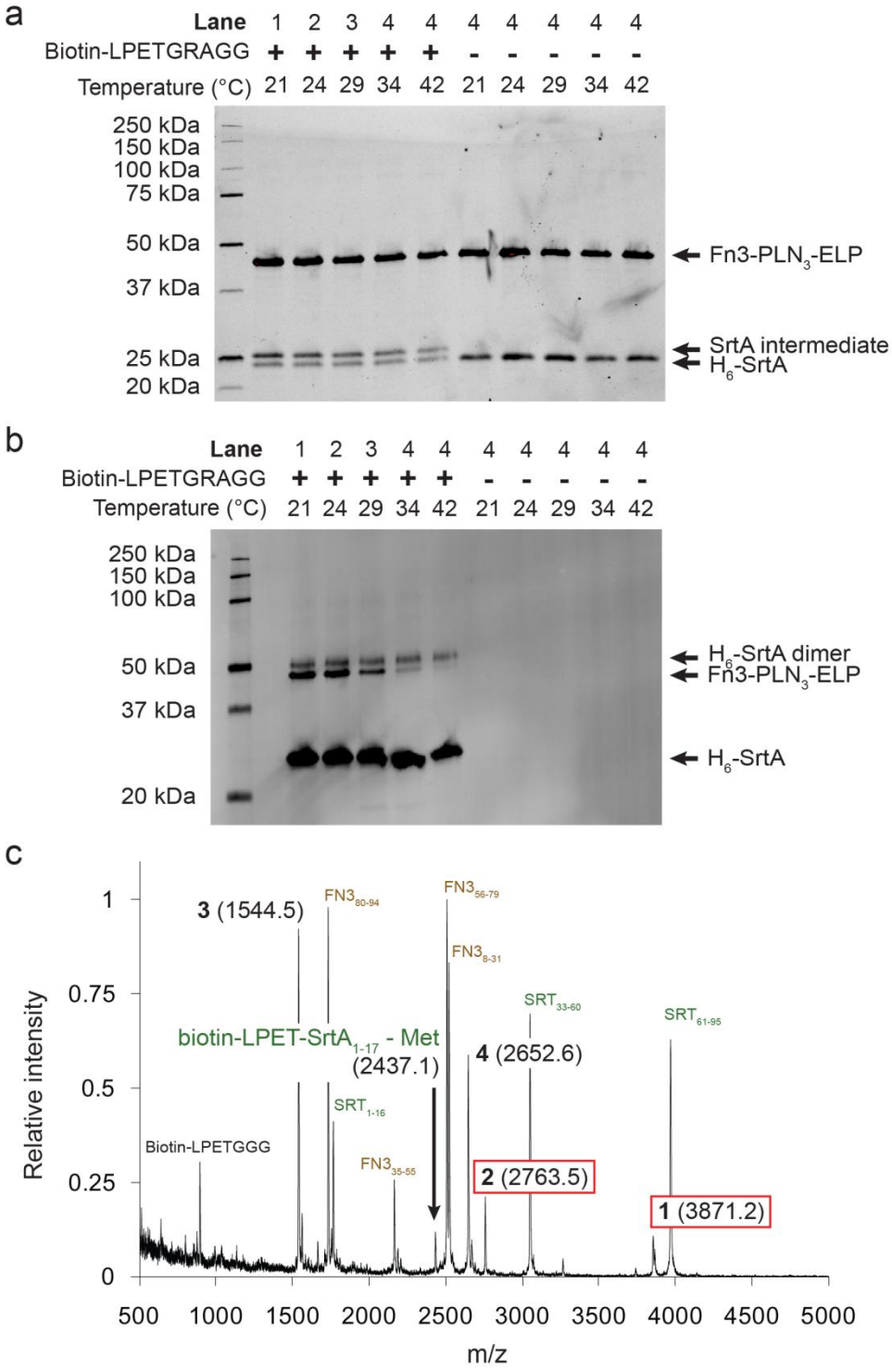
Supplementary Figure 6.



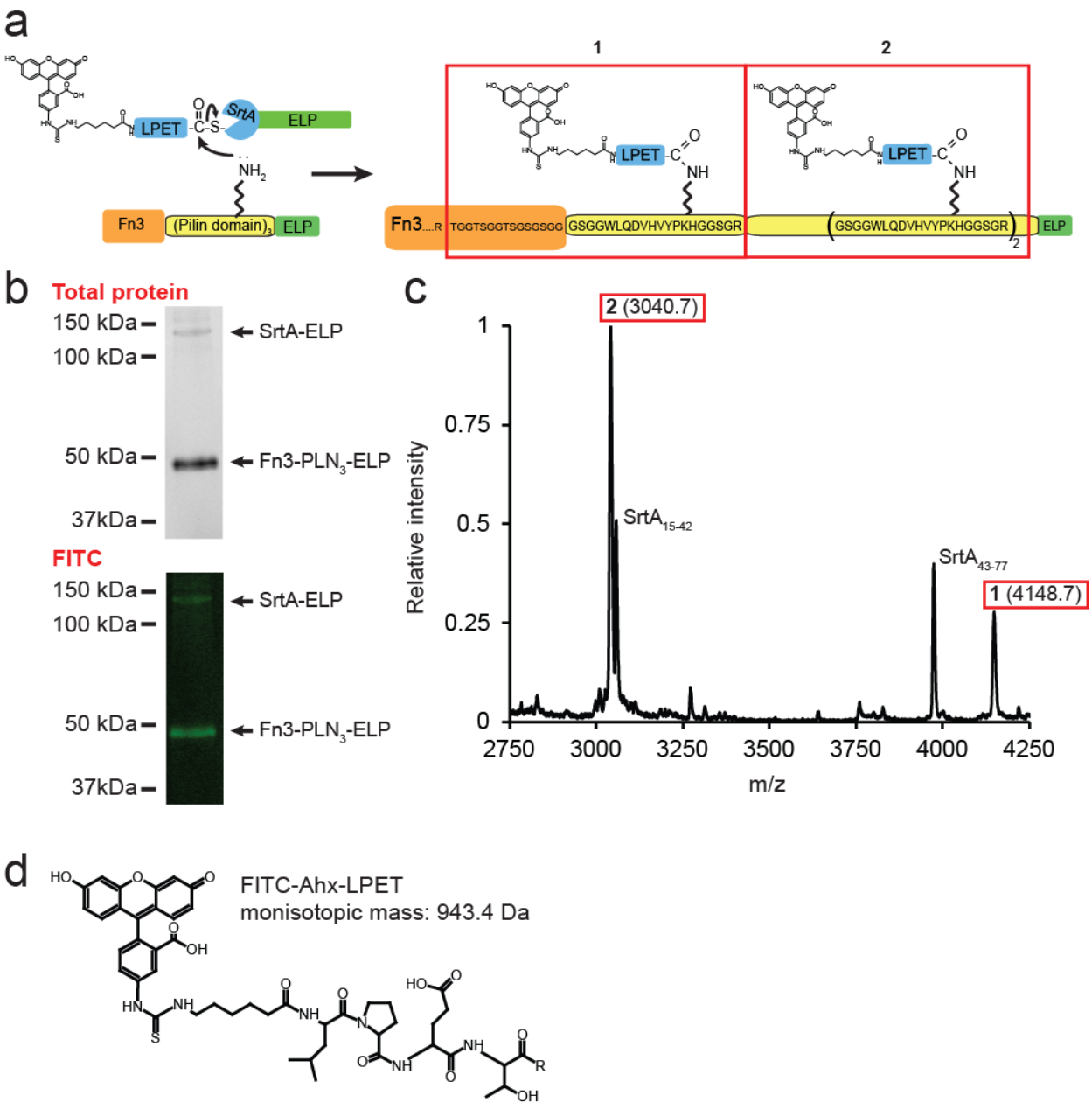
Supplementary Figure 7.

H_6-SrtA amino acid sequence

MGSSHHHHHHSSGLVPRGSHMQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATPEQLNRGVSFAEENESLDDQNI
SIAGHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRDVKPTDVGVLDEQKGKDKQLTLITCDDYNEK
TGVWEKRKIFVATEVK

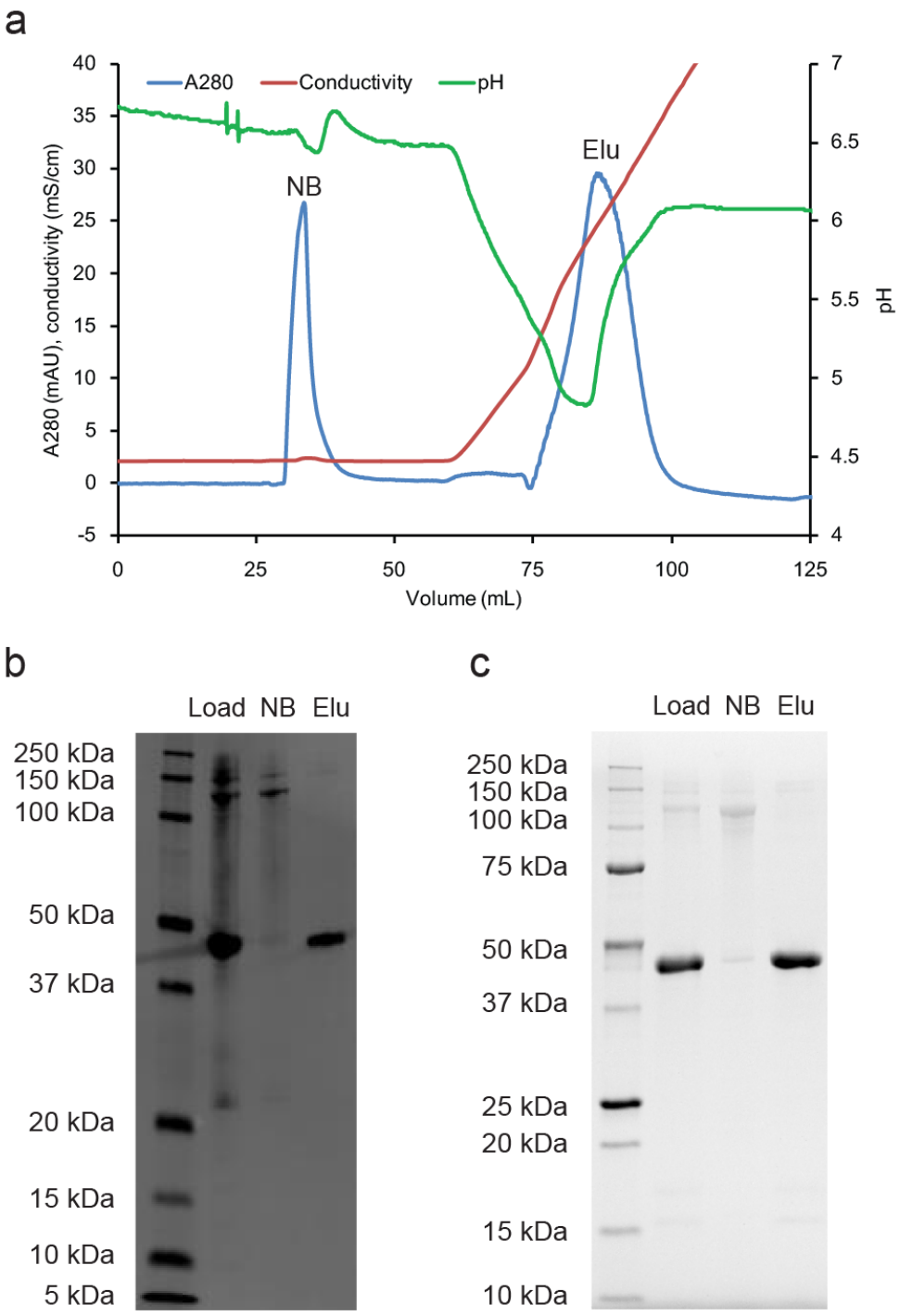


Supplementary Figure 8.

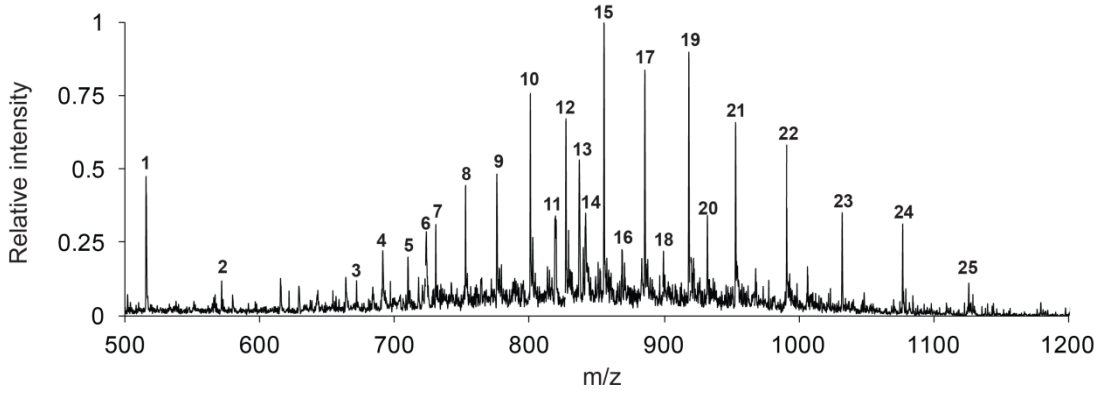


Peak Label	Peptide sequence	Predicted mass (Da)	Found mass [*m/z*-1] (Da)	Difference (Da)
1	TGGTSGGTSGSGSGGGSGGWLQDVHVYPKHGGSGR + FITC-Ahx-LPET	3203.3 + 943.4 = 4146.7	4147.7	+1.0
2	GSGGWLQDVHVYPKHGGSGR + FITC-Ahx-LPET	2095.3 + 943.4 = 3038.7	3039.7	+1.0
SRT_{15-42}	VAGYIEIPDADIKEPVYPGPATPEQLNR	3054.4	3055.0	+0.6
SRT_{43-77}	GVSFAEENESLDDQNISIAGHTFIDRPNYQFTNLK	3972.3	3973.1	+0.8

Supplementary Figure 9.

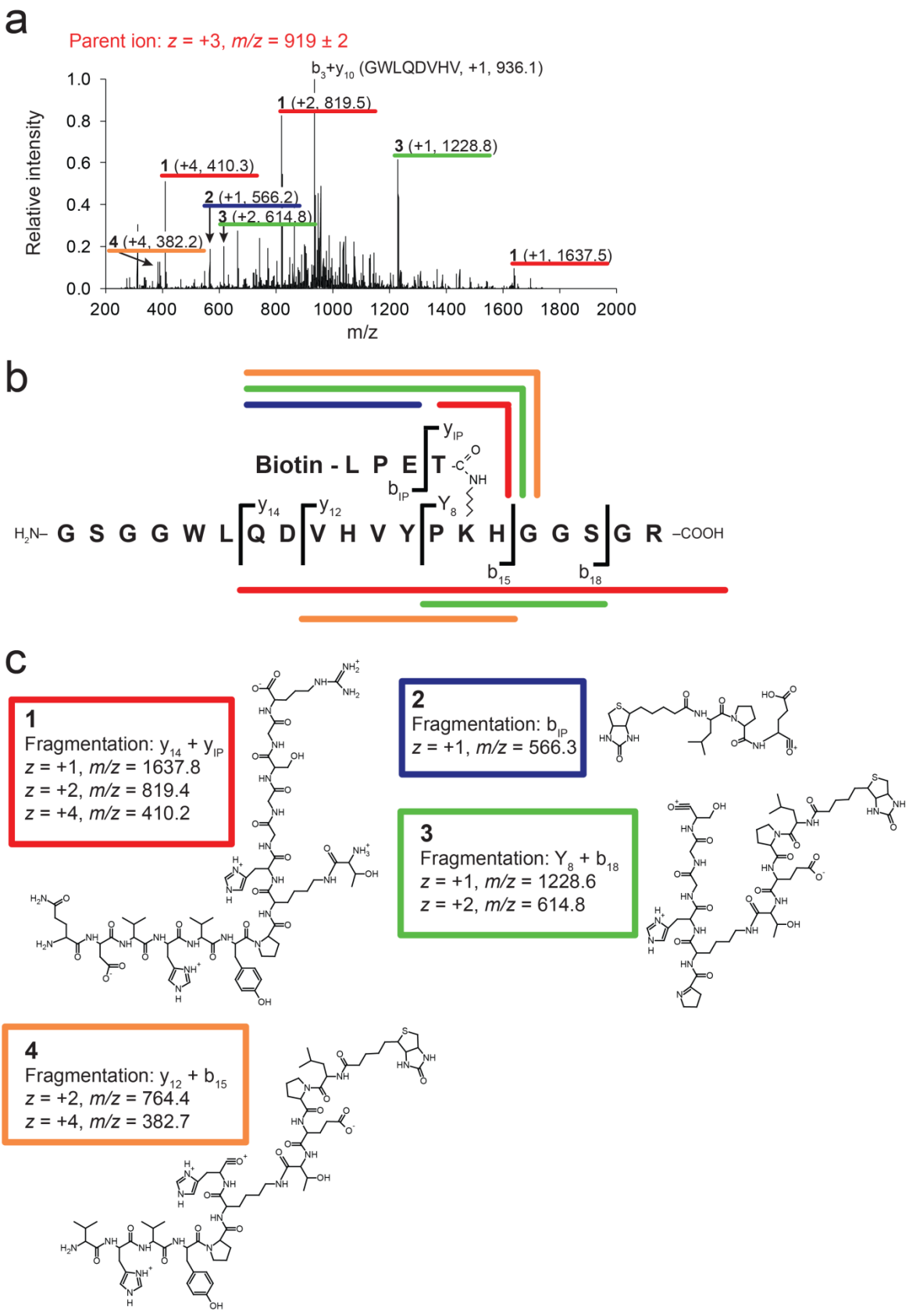


Supplementary Figure 10.

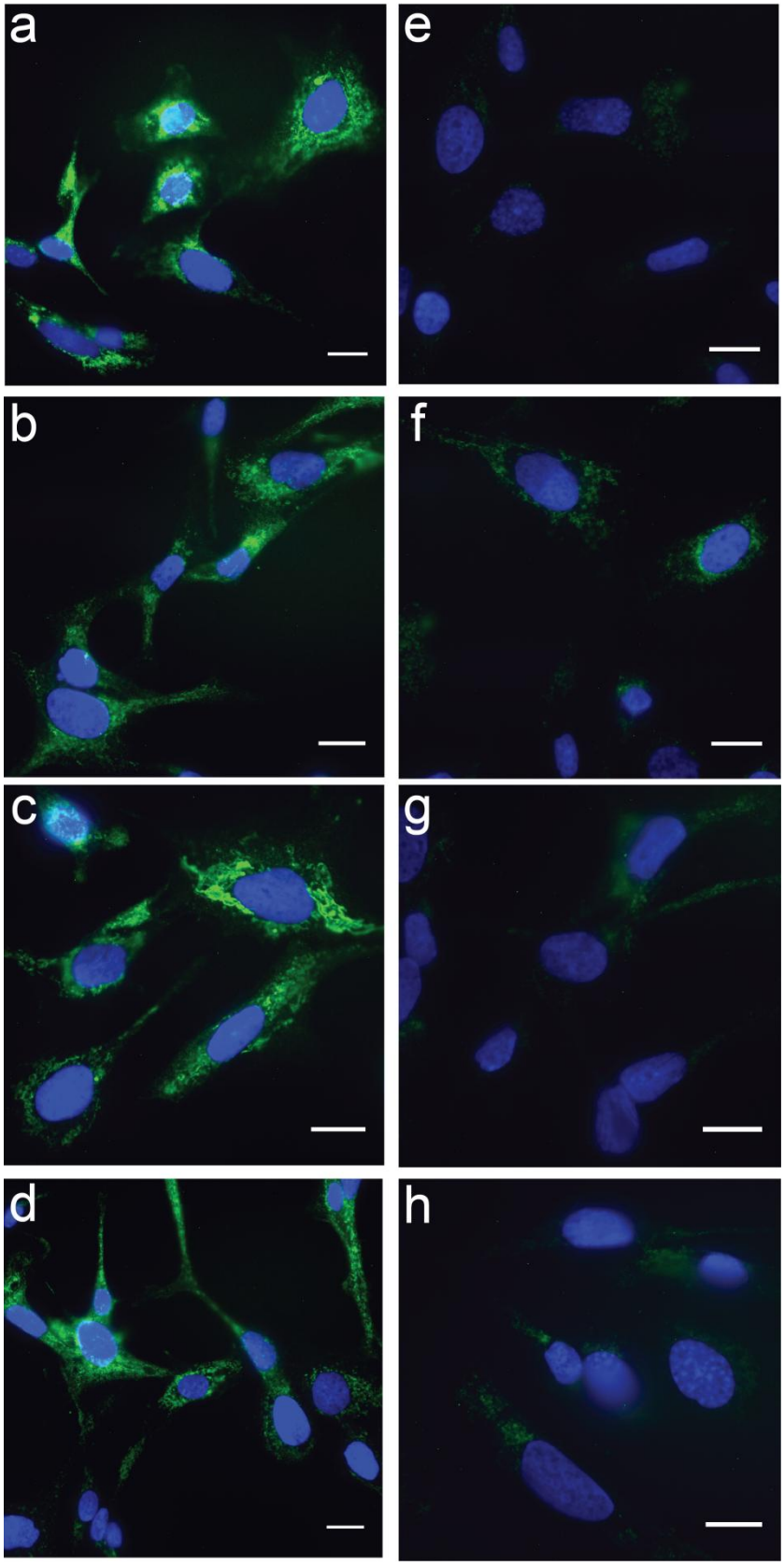


Peak label	Peptide sequence (u = lysine isopeptide-linked to biotin-LPETG)	Charge state	Predicted *m/z*	Found *m/z*
1	GSGGWLQDVHVYPK	+3	514.9	514.7
2	GDWNEGSKPISINYR	+3	579.3	579.0
3	VSDVPR (1Met loss)	+1	672.4	671.1
4	GSGGWLQDVHVYPuHGGSGR	+4	690.8	690.6
5	ITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAV TPRGDWNEGSKPISINYR	+9	709.3	709.0
6	ITYGETGGNSPVQEFTVPGSK	+3	723.4	722.9
7	GDWNEGSKPISINYRTGGTSGGTSGSGSGGGSGGWLQDVH VYPK + NH_4^+	+6	731.5	730.0
8	DLEVVAATPTSLLISWDAPAVTVRYYR	+4	752.4	752.1
9	TGGTSGGTSGSGSGGGSGGWLQDVHVYPuHGGSGR	+5	774.4	775.3
10	GDWNEGSKPISINYRTGGTSGGTSGSGSGGGSGGWLQDVH VYPuHGGSGR + NH_4^+	+7	801.1	800.2
11	MVSDVPR + oxidation at M	+1	819.4	818.5
12	TGGTSGGTSGSGSGGGSGGWLQDVHVYPuHGGSGRGSGG WLQDVHVYPuHGGSGR	+8	827.0	826.6
13	STATISGLKPGVDYTITVYAVTPR	+3	837.5	837.1
14	STATISGLKPGVDYTITVYAVTPR + NH_4^+	+3	842.8	841.2
15	TGGTSGGTSGSGSGGGSGGWLQDVHVYPuHGGSGRGSGG WLQDVHVYPuHGGSGRGSGGWLQDVHVYPuHGGSGR + 3NH_4^+	+11	855.6	855.0
16	GDWNEGSKPISINYR	+2	868.3	868.4
17	TGGTSGGTSGSGSGGGSGGWLQDVHVYPK	+3	884.1	885.2
18	TGGTSGGTSGSGSGGGSGGWLQDVHVYPuHGGSGRGSGG WLQDVHVYPK	+6	899.4	900.0
19	GSGGWLQDVHVYPuHGGSGR	+3	920.4	919.1
20	GDWNEGSKPISINYRTGGTSGGTSGSGSGGGSGGWLQDVH VYPuHGGSGR	+6	931.6	932.1
21	MVSDVPRDLEVVAATPTSLLISWDAPAVTVRYYR	+4	952.5	954
22	TGGTSGGTSGSGSGGGSGGWLQDVHVYPuHGGSGRGSGG WLQDVHVYPKHGGSGR	+6	991.3	991.2
23	DLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQ EFTVPGSK	+5	1031.9	1033.6
24	HGGSGRGSGGWLQDVHVYPuHGGSGRGSGGWLQDVH VYPKHGGSGR	+5	1076.8	1078.1
25	ITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVY AVTPRTGGTSGGTSGSGSGGGSGG WLQDVHVYPK	+8	1126.8	1127.8

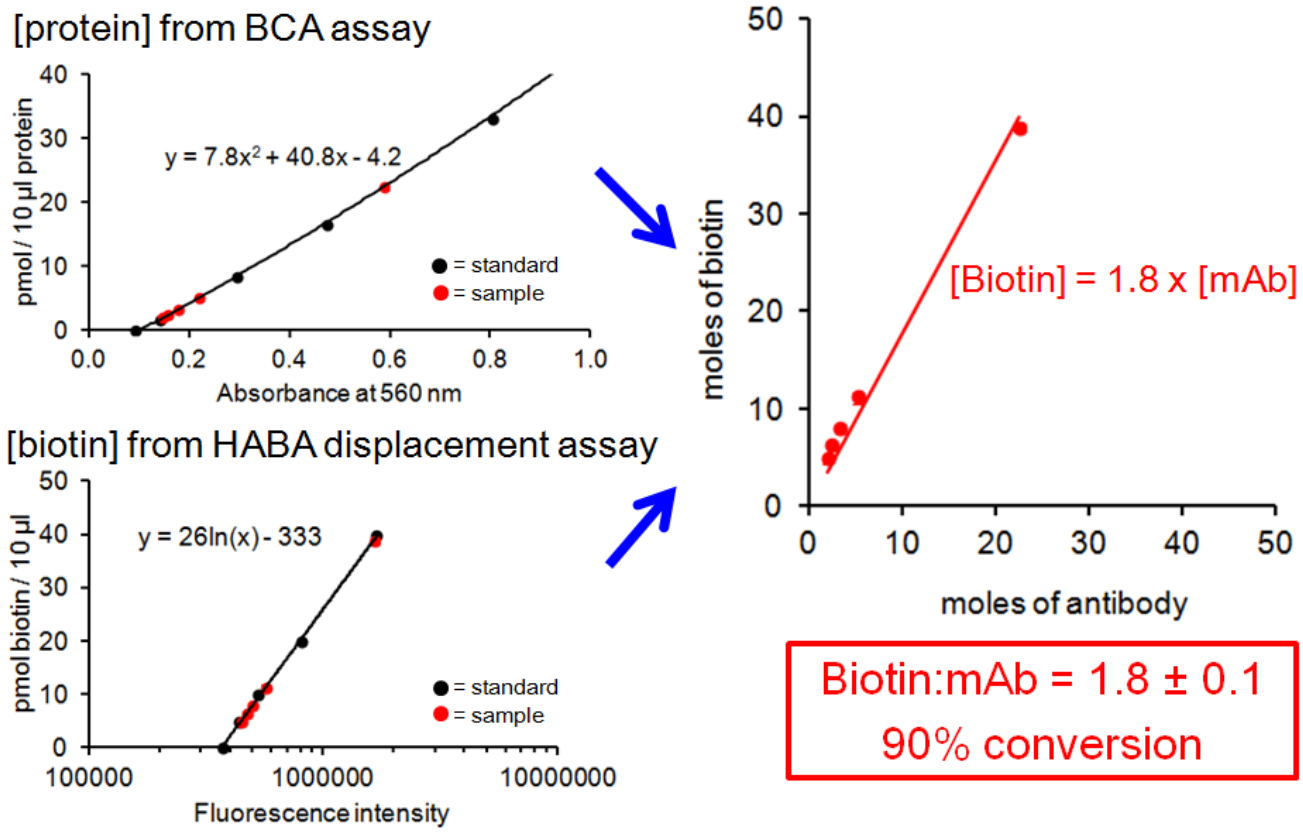
Supplementary Figure 11.



Supplementary Figure 12.



Supplementary Figure 13.



DETAILED EXPERIMENTAL SECTION

Protein expression and purification. SrtA-ELP, Fn3-ELP, and Fn3-PLN_3-ELP fusion proteins were genetically assembled in pET24b vectors, expressed in BL21 (DE3) *E. coli* (EdgeBio, Gaithersburg, MD), and purified from soluble bacterial lysate as described previously.[15]

Genes encoding the heavy and light chains of the murine IgG1 κ anti-human Her2 antibody (clone 4D5) were cloned from a hybridoma obtained through the Duke University Health System Cell Culture Facility (Durham, North Carolina). The 3' end of the heavy chain DNA was modified to contain one copy of the coding sequence for a pilin domain peptide, and the modified and unmodified antibodies were expressed by transient transfection of HEK293 cells using the Expi293 system (Life Technologies, Grand Island, NY). Secreted antibodies were purified from the culture supernatant by protein G chromatography (Thermo Scientific, Rockford, IL).

Protein-small molecule conjugation reactions. Fn3-ELP or Fn3-PLN_3-ELP were reacted at 50 μM with SrtA-ELP at 100 μM and biotin-LPETGRAGG or FITC-LPETGRAGG peptide at 500 μM in reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$). Pilin domain peptide was purchased from Genscript and was reacted at 10 μM with SrtA-ELP at 100 μM and biotin-LPETGRAGG peptide at 500 μM in reaction buffer. Purified antibodies were reacted at ~0.5 mg/ml with SrtA-ELP at 10 μM (2-fold excess over the mAb) and biotin-LPETGRAGG peptide at 500 μM (~100-fold excess over available pilin domains) in reaction buffer. All reactions were allowed to proceed overnight at 32°C. All reactions were quenched by the addition of triglycine peptide to 10 μM.

Reaction product was run on SDS-PAGE and transferred to a PVDF membrane (Bio-Rad), blocked with 5% nonfat milk, and probed with 1 mg/ml streptavidin-Cy5 conjugate (Life Technologies) at a 1:20,000 dilution in Tris-buffered saline with 1% Tween-20 and 0.5% nonfat milk. Fluorescent bands were detected with a Typhoon variable mode laser scanner (GE Healthcare Life Sciences, Pittsburgh, PA). The unpurified reaction product was trypsinized and analyzed by MALDI-TOF using α-Cyano-4-hydroxycinnamic acid matrix. The MS-Digest function of ProteinProspector v 5.10.14 (available at http://prospector.ucsf.edu/prospector/mshome.htm) was used to calculate expected tryptic peptide masses for recombinant protein sequences.

LC-MS/MS and biotin:protein molar ratio. Biotinylated Fn3-PLN_3-ELP purified by cation exchange chromatography was digested with MS-grade trypsin overnight at 20°C at a trypsin:substrate ratio of approximately 1:20 (m/m). Desalted peptides in 50 mM ammonium bicarbonate were separated by reverse-phase high pressure liquid chromatography using a Supelco Ascentis (5cmx1mm, 3μm) C18 column over an elution gradient of 5-50% buffer B (5% H_2O, 95% acetonitrile). MS data was collected using an Agilent 1100 series quadrupole ion trap (Agilent Technologies, Santa Clara, CA). For fragmentation and MS2, parent ions were isolated in the trap and fragmented by collisions with helium. Parent and daughter ions were identified using the MS-Digest and MS-Product functions of ProteinProspector v 5.10.14.

The biotin:protein ratio was assessed by comparison of the integrated areas of summed extracted ion chromatograms for ions containing either biotinylated or non-biotinylated pilin domain lysines. For Fn3-PLN_3-ELP reactions, a colorimetric quantitation kit (Thermo Scientific) that measured the decrease in HABA 500 nm absorbance when displaced from avidin by protein-bound biotin was also used according to the manufacturer's instructions. For antibody reactions, the fluorescent version of this HABA displacement assay was used per the manufacturer's instructions. Prior to analysis, biotinylated proteins were diafiltered against at least 30 diavolumes of PBS. Protein concentrations used in the assay were obtained using the bicinchoninic acid (BCA) assay. Measurements of biotin and protein concentrations were performed in triplicate for each sample and standard.

Immunofluorescence microscopy. SK-OV-3 cells were obtained from the Duke University Health System Cell Culture Facility and were maintained in McCoy's 5a medium with 10% heat inactivated fetal bovine serum, 5 units/ml penicillin, and 5 μg/ml streptomycin (Gibco). Cells were attached to glass coverslips overnight. Cells were washed in PBS, then fixed with 4% paraformaldehyde in PBS at room temperature for 15 minutes. Fixed cells were stained with a 1:200 dilution of 0.5 mg/ml anti-Her2 (with a pilin domain on each heavy chain) biotinylated by reaction with SrtA or a biotinylated murine IgG1 isotype control antibody, followed by a 1:200 dilution of 1 mg/ml streptavidin-FITC conjugate. Cells were analyzed with a Nikon TE2000-U inverted fluorescent microscope with a 60x 1.25NA oil immersion objective.