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Sortase-promoted synthesis of homooligomers from a monomeric protein

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*Materials***.** All chemicals used were obtained from commercial sources and used without further purification: 1,4-dithiothreitol (DTT), tris(hydroxymethyl)aminomethane, NaCl, sodium dodecyl sulfate (SDS) from Fluka AG (Switzerland); *N,N*-methylenebisacrylamide, acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED) from Bio-Rad (USA). Calcium independent SrtA was obtained according to the protocol [S1]. PrtA construct was obtained according to the protocol [S2]; the construct of the used recombinant protein A in block form looks like this:

Linker ~ Domain B of PrtA ~ domain B of PrtA ~ LPETGEAHHHHHHGC

That is, the construct contains linker, two copies of protein A itself (it is common practice to link two to six copies), an LPETG pentapeptide specific for enzyme recognition, and a histidine tag to facilitate isolation of the construct. The complete sequence is given in [S2].

The initial concentration of proteins were determined spectrophotometrically using the extinction coefficient calculated in the ProtParam tool program (extinction coefficient ε_{280}=4470 $M^{-1}cm^{-1}$ for PrtA; extinction coefficient ε_{280}=17420 $M^{-1}cm^{-1}$ for SrtA).

Enzymatic reactions were carried out in 100 µl of buffer (50 mM Tris, 2 mM DTT, 0.1 M NaCl, pH=8.0) at 37 C for 3 hours. The concentration of protein A in the reaction mixture was 0.4 mg/ml (26 µM), sortase was introduced in a molar ratio of 1:10 (enzyme: protein), 1:1 or 1:10 by moles.

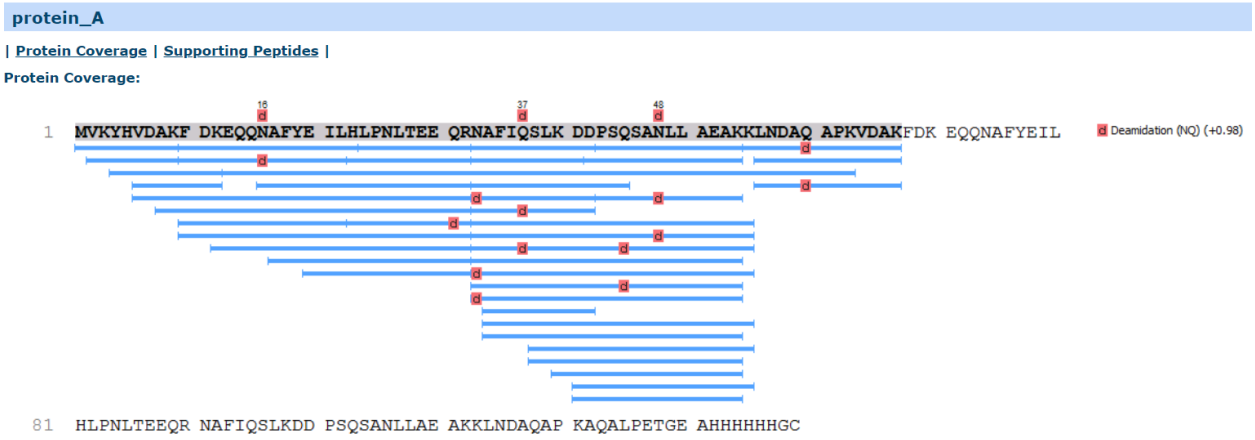
Characterization of protein conjugates. Electrophoresis was performed according to Laemmli (SDS-PAGE in 12% gel), all prepared samples were boiled in the presence of SDS and 2-mercaptoethanol. Gels were stained with Coomassie G-250 (Bio-Rad). Calculation of the bands intensity ratio of the obtained conjugates after staining was carried out using the program GelAnalyzer 19.1. In-gel digestion of proteins was performed as was described previously [S3] with minor modifications. Briefly, excised

protein bands were cut into cubes (ca. 1 x 1 mm), destained with 50% acetonitrile in 200 mM ammonium bicarbonate for 30 min, dehydrated with neat acetonitrile for 15 min and dried using SpeedVac vacuum concentrator (Savant) for 10 min. For in-gel digestion of protein trypsin buffer (50 mM ammonium bicarbonate solution contained 15 ng/ml trypsin) was added to gel pieces and sample was incubated overnight at 37°C in air circulating thermostat. 120 µl of 0.2% TFA in water (vol/vol) were added to the sample and incubated for 1.5 hour at room temperature to extract peptides. Peptide solution was transferred in a clean tube, vacuum dried and stored at -80°C. Peptides were dissolved in 15 µl of loading solution (2% acetonitrile, 98% H_2O, and 0.1% TFA) before analysis.

MS raw files were analyzed by Peaks studio 10.0 (Bioinformatics Solutions Inc.) [S4]. Identification of protein was made by searching against the sequence of target protein with deamidation Asn/Gln and Met oxidation as variable modifications. False discovery rate for peptide-spectrum matches was determined by searching a reverse database and was set to 0.01. Enzyme specificity was set as C-terminal to arginine and lysine, and a maximum of three missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 10 p.p.m. and an allowed fragment mass deviation 0.05 Da.

Proteomic analysis of the monomer PrtA-LPET-T14

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	Coverage (%) Sample 1	Area Sample 1	#Peptides	#Unique	#Spec Sample 1	PTM	Avg. Mass	Description
1	19	protein_A	223.92	48	48	4.3321E9	33	33	63	Y	15762	protein_A



Proteomic analysis of the dimer (PrtA-LPET)$_2$-T14

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	Coverage (%) Sample 2	Area Sample 2	#Peptides	#Unique	#Spec Sample 2	PTM	Avg. Mass	Description
1	19	protein_A	109.47	27	27	3.2235E8	4	4	4	N	15762	protein_A

protein_A

| Protein Coverage | Supporting Peptides |

Protein Coverage:

```
  1  MVKYHVDAKF DKEQQNAFYE ILHLPNLTEE QRNAFIQSLK DDPSQSANLL AEAKKLNDAQ APKVDAKFDK EQQNAFYEIL

 81  HLPNLTEEQR NAFIQSLKDD PSQSANLLAE AKKLNDAQAP KAQALPETGE AHHHHHHGC
```

Proteomic analysis of the trimer (PrtA-LPET)$_3$-T14

1	19	protein_A	100.77	32	32	6.9347E7	7	7	12	Y	15762	protein_A

protein_A

| Protein Coverage | Supporting Peptides |

Protein Coverage:

```
  1  MVKYHVDAKF DKEQQNAFYE ILHLPNLTEE QRNAFIQSLK DDPSQSANLL AEAKKLNDAQ APKVDAKFDK EQQNAFYEIL   d Deamidation (NQ) (+0.98)

 81  HLPNLTEEQR NAFIQSLKDD PSQSANLLAE AKKLNDAQAP KAQALPETGE AHHHHHHGC
```

Supporting Peptides:

Proteomic analysis of the monomer PrtA-LPET-T17

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	Coverage (%) Sample 4	Area Sample 4	#Peptides	#Unique	#Spec Sample 4	PTM	Avg. Mass	Description
1	19	protein_A	204.04	47	47	2.7223E9	22	22	36	Y	15762	protein_A

protein_A

| Protein Coverage | Supporting Peptides |

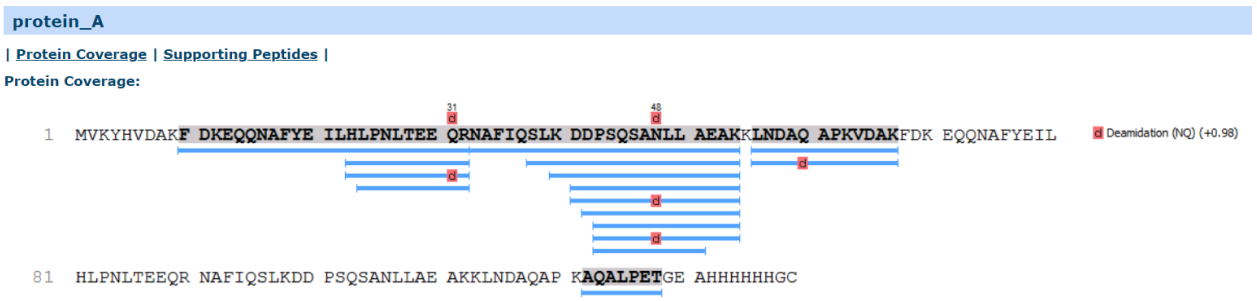
Protein Coverage:

```
  1  MVKYHVDAKF DKEQQNAFYE ILHLPNLTEE QRNAFIQSLK DDPSQSANLL AEAKKLNDAQ APKVDAKFDK EQQNAFYEIL   d Deamidation (NQ) (+0.98)

 81  HLPNLTEEQR NAFIQSLKDD PSQSANLLAE AKKLNDAQAP KAQALPETGE AHHHHHHGC
```

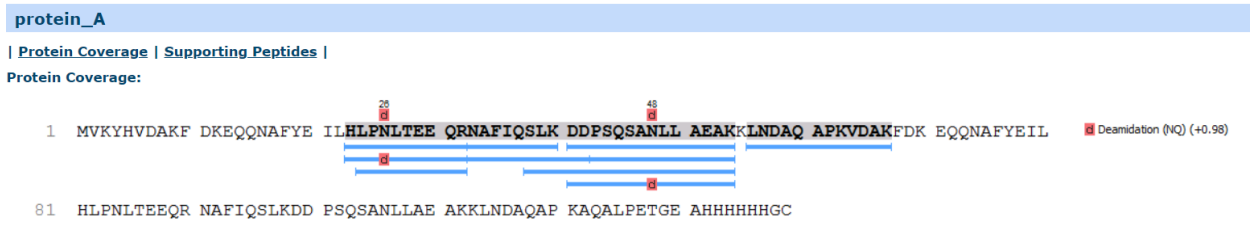
Proteomic analysis of the dimer (PrtA-LPET)$_2$-T17

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	Coverage (%) Sample 5	Area Sample 5	#Peptides	#Unique	#Spec Sample 5	PTM	Avg. Mass	Description
1	19	protein_A	131.20	46	46	4.1245E8	12	12	22	Y	15762	protein_A



Proteomic analysis of the trimer (PrtA-LPET)$_3$-T17

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	Coverage (%) Sample 6	Area Sample 6	#Peptides	#Unique	#Spec Sample 6	PTM	Avg. Mass	Description
1	19	protein_A	126.81	32	32	3.4848E8	8	8	11	Y	15762	protein_A

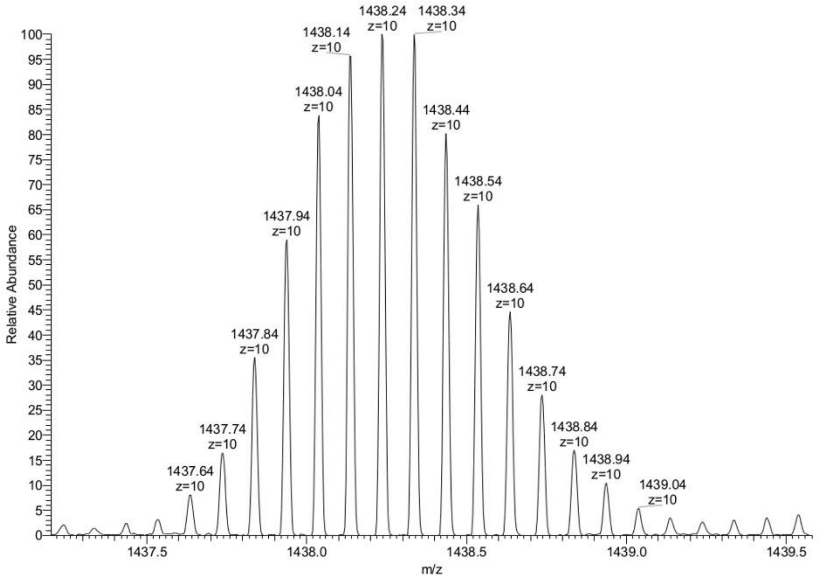


Liquid chromatography/mass spectrometry

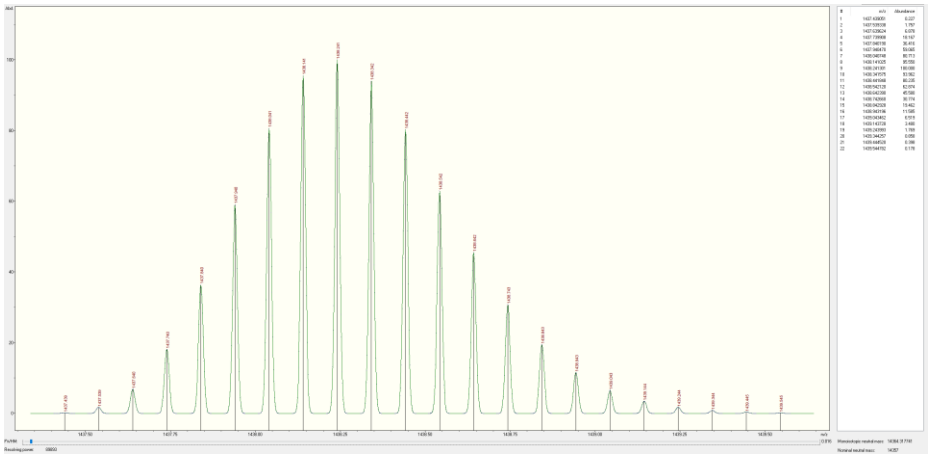
Samples were loaded to a home-made trap column 50x0.1 mm, packed with Prontosil 120-C18AQ 5µm sorbent, in the loading buffer (2% MeCN, 98% H_2O, 0.1% TFA) at 4 µl/min flow and separated at RT in a home-packed [S5] fused-silica column 300x0.1 mm packed with Reprosil PUR C18AQ 1.9 into an emitter prepared with P2000 Laser Puller (Sutter, USA). Reverse-phase chromatography was performed with an Ultimate 3000 Nano LC System (Thermo Fisher Scientific), which was coupled to the Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray source (Thermo Fisher Scientific). Peptides were loaded in a loading solution (98% 0.1% (v/v) formic acid, 2% (v/v) acetonitrile) and eluted with a linear gradient: 5% B for 3 min; 5-50% B for 15 min, 50% B for 4 min, 50-80% B for 1 min, 80% B during 3 min, 80-5% B for 1 min at a flow rate of 500 nl/min. Buffer A was 5% acetonitrile

and 0.1% formic acid and buffer B was 80% acetonitrile and 0.1% formic acid. MS1 parameters were as follows: 70K resolution, 350-1600 scan range, max injection time – 35 msec, AGC target – $3x10^6$. Ions were isolated with 1.4 m/z window, preferred peptide match and isotope exclusion. Dynamic exclusion was set to 30 s. MS2 fragmentation was carried out in HCD mode at 17.5K resolution with HCD collision energy 30%, max injection time – 80 msec, AGC target – $1x10^5$.

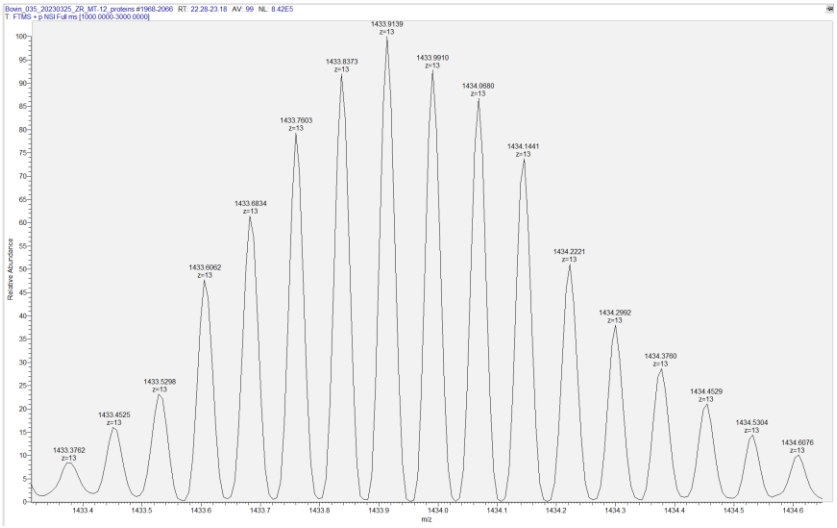
Mass-spectra



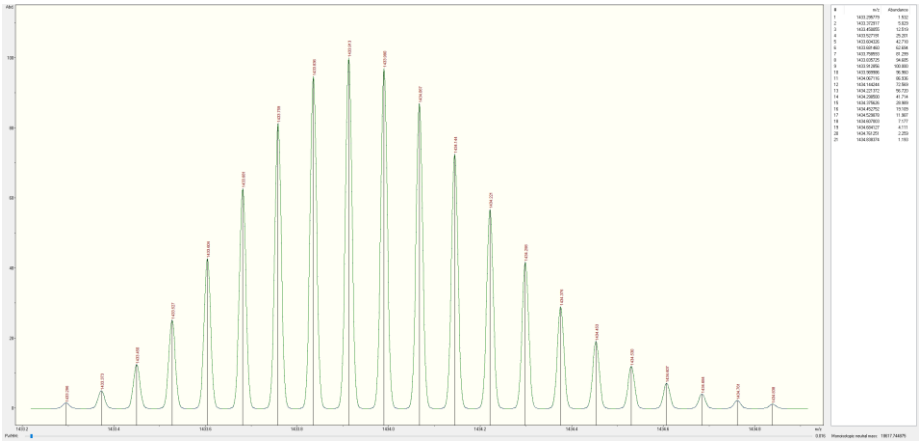
PrtA-LPET, Mf = $C_{642}H_{1010}N_{176}O_{206}S$, PrtA-LPET minus Methionine minus H_2O Mf = $C_{637}H_{999}N_{175}O_{204}$. Monoisotopic mass: found m/z 1437.64; calc. for $C_{637}H_{999}N_{175}O_{204}$ $[M+10H]^{10+}$ 1437.44.



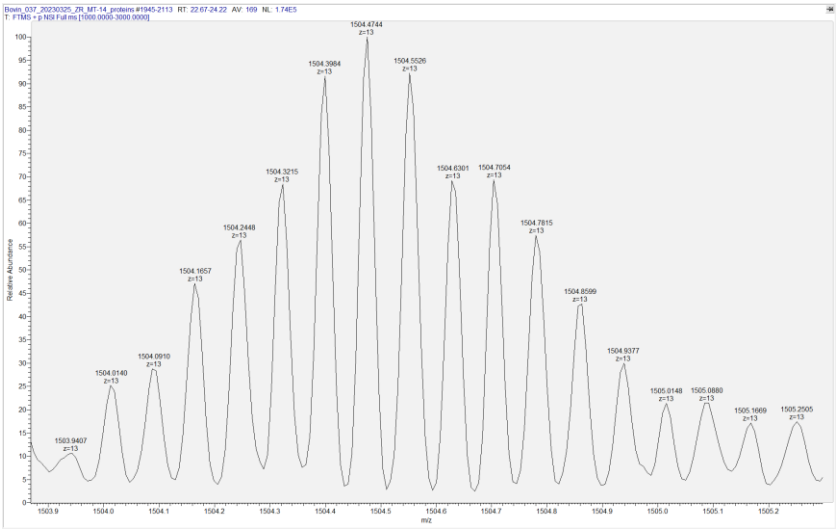
Theoretical mass spectrum of the PrtA-LPET minus Methionine minus H_2O Mf = $C_{637}H_{999}N_{175}O_{204}$. Monoisotopic mass calc. for $C_{637}H_{999}N_{175}O_{204}$ $[M+10H]^{10+}$ 1437.44.



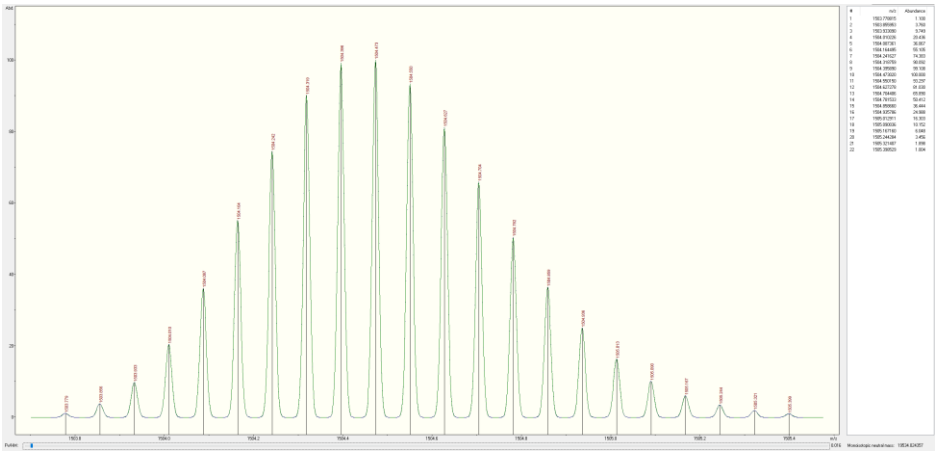
PrtA-LPET-T14 monomer. Mf = $C_{791}H_{1224}N_{236}O_{293}S$, PrtA-LPET-T14 minus Methionine Mf = $C_{786}H_{1215}N_{235}O_{292}$. Monoisotopic mass: found m/z 1433.376; calc. for $C_{786}H_{1215}N_{235}O_{292}$ $[M+13H]^{13+}$ 1433.296.



Theoretical mass spectrum of the monomer PrtA-LPET-T14 minus Methionine. Monoisotopic mass calc. for $C_{786}H_{1215}N_{235}O_{292}$ $[M+13H]^{13+}$ 1433.296.



PrtA-LPET-T17 monomer. Mf = $C_{823}H_{1268}N_{248}O_{313}S$, PrtA-LPET-T17 minus Methionine Mf = $C_{818}H_{1259}N_{247}O_{312}$. Monoisotopic mass: found m/z 1503.941; calc. for $C_{818}H_{1259}N_{247}O_{312}$ $[M+13H]^{13+}$ 1503.779.



Theoretical mass spectrum of the monomer PrtA-LPET-T17 minus Methionine. Monoisotopic mass calc. for $C_{818}H_{1259}N_{247}O_{312}$ $[M+13H]^{13+}$ 1503.779.

References

S1. H. Hirakawa, S. Ishikawa and T. Nagamune, *Biotechnol. J.,* 2015, **10**, 1487, doi:10.1002/biot.201500012.

S2. V. N. Stepanenko, T. D. Melikhova, B. Z. Eletskaya, I. V. Myagkikh and M. N. Tereshin, *Patent RU 2789032*, 2023.

S3. A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen and M. Mann, *Nat. Protoc.,* 2006, **1**, 2856.

S4. B. Ma, K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty-Kirby and G. Lajoie, *Rapid Commun. Mass Spectrom.*, 2003. **17**, 2337.

S5. S. I. Kovalchuk, O. N. Jensen and A. Rogowska-Wrzesinska, *Mol. Cell Proteomics,* 2019, **18**, 383.