Supporting Information

Partially Shielded Enzymes Capable of Processing Large Protein Substrates

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Materials and methods

Chemicals Tetraethyl orthosilicate (**T**, ≥ 99%), (3-aminopropyl)triethoxysilane (**A**, ≥ 98%), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), glutaraldehyde (grade I, 25% in water), sodium chloride (NaCl, ≥ 99.0%) and Trizma® base (≥ 99.9%) were purchased from Sigma-Aldrich (Switzerland). Potassium phosphate salts were purchased from Fisher Scientific (Switzerland). $CaCl_2$ was purchased from Carl Roth (Germany). Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Switzerland). Precast protein gels (4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µl) and Ladder (Precision Plus Protein™ Dual Xtra Prestained Proteins Standards) were purchased from Bio-Rad. SensoLyte® 520 Sortase A Activity Assay Kit was purchased from AnaSpec (USA). Gly_5-modified Fluorescein isothiocyanate (FITC) was purchased from Bachem (Switzerland). Sortase A (SrtA) mutant and CD30-specific cAC10/brentuximab were produced as described previously.[1] Recombinant non-methylated porcine trypsin (EC 3.4.21.4), MS grade, was purchased from Eucodis Bioscience (Austria).

Buffer solutions Buffer solutions were prepared with nanopure water. KPi: 50 mM potassium phosphate, 100 mM NaCl, pH 8.0. Borate buffer: 100 mM sodium borate buffer, pH 9.0. Tris buffer: 50 mM Tris base, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5.

Equipment Activity assays and bioconjugation reactions were performed in a Thermomixer (Thermomixer Comfort, Eppendorf AG, Germany) in the dark. 5-FAM fluorescence was monitored using a Synergy H1 (BioTek, Switzerland) into 96-well plate (Microplatte 96 Well Half Area, Huber Lab, Switzerland). Nanopure water (resistivity ≥18 MΩ cm) was produced with a Millipore ®Synergy purification system (Merck, Switzerland). SDS-PAGE gels were carried out using a PowerPac™ basic power supply (BioRad, Switzerland). SDS-PAGE gels were analysed with an image scanner Fujifilm FLA-9000 (USA). SDS-PAGE gels were stained using a staining solution (Bio-Safe™ Coomassie G-250 Stain) from Bio-Rad (Switzerland). Particles were imaged using a Zeiss SUPRA® 40VP scanning electron microscope (Germany). Particle sizes were measured using the ®AnalySIS software package. Digested cell lysates were analysed by loading onto a Pepmap100 Trap column (C_{18}, 5 µm, 100A, 300 µm x 5 mm, Thermo Fisher Scientific, Switzerland) and separated by back flush onto an analytical column (C_{18}, 3 µm, 100A, 75 µm x 150 mm, Nikkyo Technos, Japan). MS analysis was performed with QExactive classic mass spectrometer (Thermo Fisher Scientific, Switzerland).

Experimental Procedures

Amino modification of SPs. SPs were produced as previously described[2] and diluted in 200 mL H_2O to obtain a solution of [SPs] = 3.2 mg/mL. **A** (0.555 mmol) was added to the previous solution and the reaction mixture was stirred at 20°C, 400 rpm for 90 min. Subsequently, the suspension was centrifuged at 4000 rpm for 15 min and the supernatant was removed.The particles were resuspended in distilled H_2O (50 mL) and submitted to ultrasonic treatment. This washing step was repeated twice. The particles were then resuspended in H_2O (50 mL). SPs-NH_2 (11.3 mg/mL) were stored at 4°C.

Glutaraldehyde cross-linking To a suspension of SPs-NH_2 (3.0 mL, 10.4 mg/mL) was added glutaraldehyde (0.098 mmol). The reaction mixture was stirred at 20°C, 400 rpm for 30 min. Then, the suspension was washed twice in H_2O, sonicated and resuspended in 3 mL KPi to yield SPs-GA (10.4 mg/mL).

Sortase Immobilisation To SPs-GA (2.6 mL, 10.4 mg/mL) was added 5.9 mL of KPi to reach [SPs-GA]=3.2 mg/mL. SrtA (final concentration of 45 µg/mL) was added to the suspension and the resulting mixture was stirred at 20°C, 400 rpm for 1 h to produce SPs-SrtA. A BCA assay was performed on the supernatant collected after protein immobilisation and showed that 81% of the enzyme was immobilised at the surface of SPs-GA.

OD	OD-Blank	[SrtA] in supernatant (µg/mL)	[SrtA] initial (µg/mL)	[SrtA] immobilized (µg/mL)	Average [SrtA] immobilized (µg/mL)	Average [SrtA] immobilized (µg/mg)
0.077	0.009	8.2	45	36.8	36.5	11.4
0.078	0.01	9.1	45	35.9		
0.077	0.009	8.2	45	36.8		

Trypsin Immobilisation SPs-GA (5 mL, 16 mg/mL) were centrifuged and resuspended in 5 mL of Borate buffer. The resulting suspension was incubated with trypsin (final concentration of 38 µg/mL) at 20 °C, 400 rpm for 1 h to produce SPs-Try. A BCA assay was performed on the supernatant collected after protein immobilisation and showed that 95% of the enzyme was immobilised at the surface of SPs-GA. The particle suspension was centrifuged at 350 rcf for 5 min and the particles were resuspended in 100 mM Kpi (pH 8) at a final concentration of 3.2 mg/mL.

OD	OD-Blank	[Try] in supernatant (µg/mL)	[Try] initial (µg/mL)	[Try] immobilized (µg/mL)	Average [Try] immobilized (µg/mL)	Average [Try] immobilized (µg/mg)
0.097	0.002	1.8	38.2	36.4	36.4	2.3
0.097	0.002	1.8	38.2	36.4		
0.097	0.002	1.8	38.2	36.4		

Sortase Shielding SPs-SrtA (8 mL, 3.2 mg/mL) was incubated with **T** (0.172 mmol) at 10°C, 400 rpm for 1 h. Then, **A** (0.039 mmol) was added to the reaction media. The resulting mixture was stirred at 10°C, 400 rpm for 4 h. Aliquots of 2 mL were collected every hour. The particles were centrifuged at 350 rcf for 5 min, washed in KPi and resuspended in 2 mL KPi ([SPs-SrtA-AT] = 3.2 mg/mL). The nanobiocatalysts produced were stored at room temperature for 12 hours and finally stored at 4°C.

Trypsin Shielding SPs-Try (110 mL, 3.2 mg/mL) was incubated with **T** (1.19 mmol) at 20°C, 400 rpm for 1 h. Successively, **A** (0.282 mmol) was added and the particles suspension was reacted for 5 h at 10°C, 400 rpm. The suspension produced was centrifuged at 350 rcf for 5 min and the particles were washed in 100 mM Tris buffer (pH 8) and resuspended in the same buffer at a final concentration of 16 mg/mL. The particles produced were stored at room temperature for 12 hours to allow for curing and finally stored at 4°C.

Activity Assay of the partially shielded sortase In a typical experiment, FRET sortase substrate (0.4 mM, 5-FAM/QXLTM520 Sortase substrate) was diluted 100-fold in Tris buffer. SPs-SrtA-AT (60 µL, 3.2 mg/mL) were centrifuged and resuspended in 60 µL Tris buffer. FRET sortase substrate solution (4 µM, 60 µL) was added to the SPs-SrtA-AT suspension. The resulting mixture was shaken in a thermomixer in the dark at 25°C, 1300 rpm for 30 min. After centrifugation, 100 µL of the supernatant was transferred into a black 96-well plate. 5-FAM fluorescence was monitored at excitation /emission= 490/520 nm. Cleavage rates of the quenched FRET substrate using SPs-SrtA and SPs-SrtA-AT of different layer thickness are reported in Table S1.

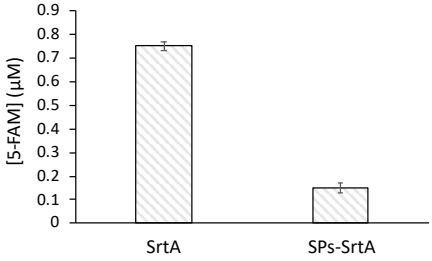


Fig. S 1 Activity of soluble SrtA and immobilized SrtA (SPs-SrtA)

Table S 1. Cleavage rates of the quenched FRET substrate using SPs-SrtA and SPs-SrtA-AT of different layer thickness

	SPs-SrtA	SPs-SrtA-AT (1h)	SPs-SrtA-AT (2h)	SPs-SrtA-AT (3h)	SPs-SrtA-AT (4h)
U ($nM.min^{-1}$)	0.40 ± 0.06	1.40 ± 0.03	3.90 ± 0.03	4.20 ± 0.02	2.90 ± 0.01

Table S2. Stability of lyophilised SPs-Srt-AT expressed as a fraction of the initial activity, in %

	SPs-SrtA-AT		
Storage temperature (°C)	-20	4	20
Time (days)			
4	105 ± 20	113 ± 20	117 ± 20
8	104 ± 20	110 ± 20	115 ± 20
13	89 ± 20	95 ± 20	98 ± 20
214	109 ± 20	101 ± 20	80 ± 20

Table S3. Stability of lyophilised SrtA expressed as a fraction of the initial activity, in %

	Free SrtA		
Storage T°C	-20	4	20
Time (days)			
1	105 ± 20	93 ± 20	97 ± 20
41	101 ± 20	97 ± 20	83 ± 20
106	108 ± 20	1 ± 20	12 ± 20

Bioconjugation reaction To a suspension of SPs-SrtA-AT ([SrtA]= 3 µM) in Tris buffer was added mAbs (10 µM) and Gly_5-FITC (200 µM). The resulting mixture was shaken in a thermomixer in the dark at 25°C, 1300 rpm for 4 h. In order to stop the bioconjugation reaction, the supernatants were transferred into new tubes after centrifugation.

SDS-PAGE of bioconjugation products 30 µL of each supernatant was mixed with 10 µL of SDS-PAGE sample buffer and boiled for 5 min. 10 µL of each resulting mixture were loaded on 4-20% precast SDS-PAGE gels. The gels were run at 150 V for 60 min. Fluorescence scans and Coomassie of the gels are shown in Fig. S1.

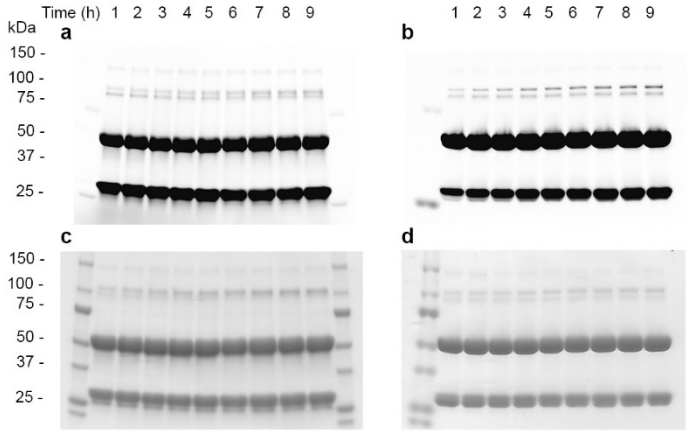
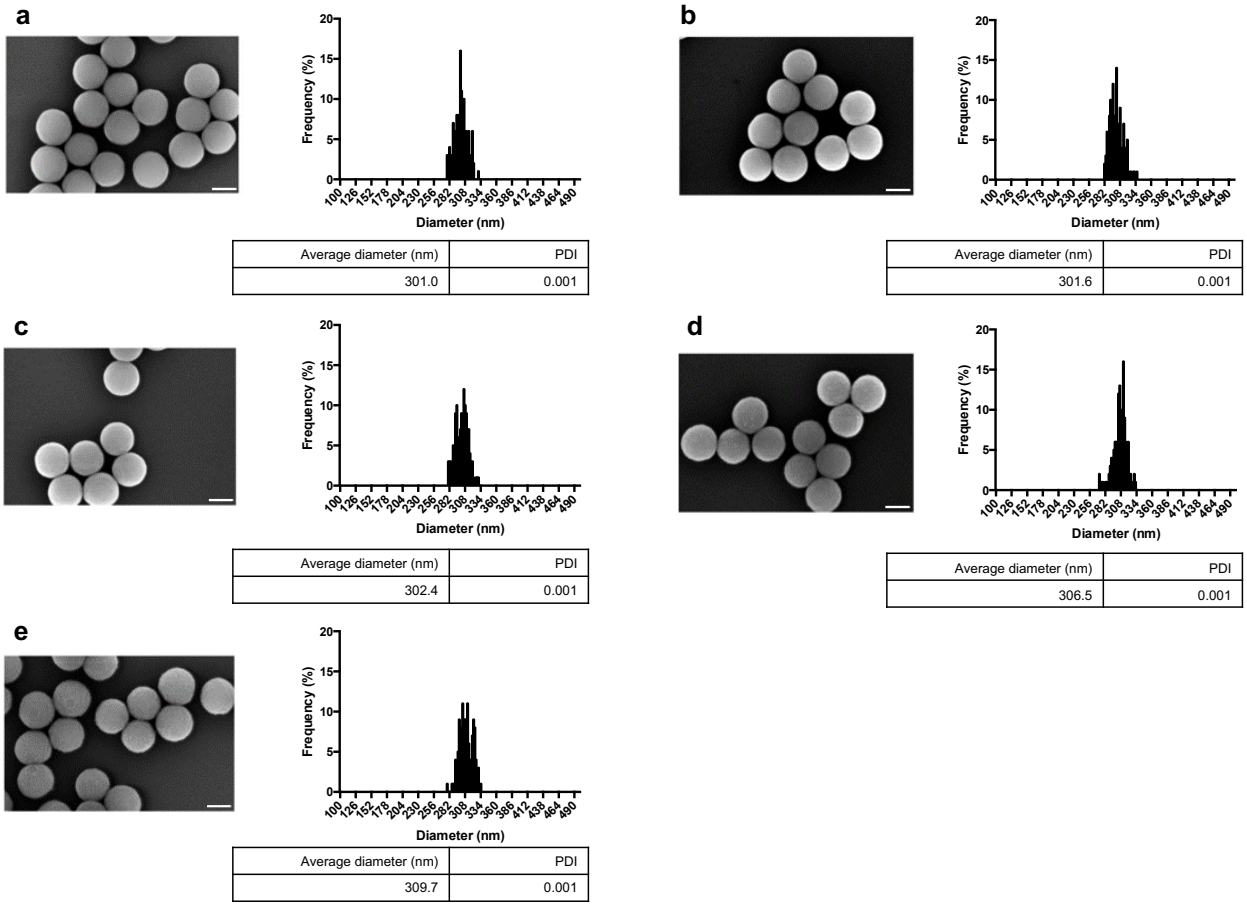


Fig. S 2 Sortase-mediated FITC conjugation using Free SrtA and SPs-SrtA-AT after 3 h incubation with **A** and **T**. **a)** Fluorescence scan at λ_{ex} = 495 nm of the reaction products using free SrtA and **c)** Coomassie of the stained gel. **b)** Fluorescence scan at λ_{ex} = 495 nm of the reaction products using SPs-SrtA-AT (3 h) and **d)** Coomassie of the stained gel.

Scanning Electron Microscopy A solution of SPs-SrtA-AT in nanopure water was prepared and spread on a silicon substrate. The sample was dried and sputter-coated with gold for 15 s at 20 mA. Secondary electron micrographs were acquired using the InLens mode with an accelerating voltage of 10 kV at a magnification of 150,000 X. Particle sizes were measured on the acquired micrographs using the ®AnalySIS software package. 100 measurements, at least, were performed for each sample (Table S4). Statistical analysis of micrographs are shown in Fig. S2.



Average diameter (nm)	PDI
301.0	0.001

Average diameter (nm)	PDI
301.6	0.001

Average diameter (nm)	PDI
302.4	0.001

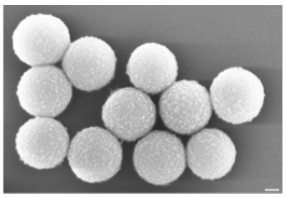
Average diameter (nm)	PDI
306.5	0.001

Average diameter (nm)	PDI
309.7	0.001

Fig. S 3 Statistical analysis of SEM micrographs of bare SPs (**a**) and SPs-SrtA-AT after 1 (**b**), 2 (**c**), 3 (**d**) and 4 (**e**) hours of reaction with **A** and **T**. Scale bars represent 200 nm.

Table S 4. Summary of Layer Growth

Reaction time with **A** and **T** (hours)	0	1	2	3	4
Average diameter (nm)	301.0	301.6	302.4	306.5	309.7
Thickness layer (nm)	0	0.3	0.7	2.7	4.3



Diameter bare SPs (nm)	Diameter SPs-Try-AT (nm)	Layer thickness of SPs-Try-AT (nm)	PDI
379.8	386.6	3.4	0.00053892

Fig. S 4 SEM micrograph and statistical analysis of SPs-Try-AT. Scale bar represents 100 nm.

Human embryonal kidney cancer cells (HEK293 cell line) HEK cells were grown to a large number of cells. Cells were washed free from culture medium and aliquots of $5x10^6$ cells were frozen. At start of evaluation period, two aliquots were defrosted, proteins lysed in 8 M urea/100 mM Tris-HCl pH 8.0 using a probe sonicator, and reduced/alkylated with DTT and iodoacetamide. Proteins were cleaned by precipitation in cold acetone. The protein pellets were reconstituted in 0.1 mL 8 M urea/50 mM Tris-HCl pH 8.0 and protein content measured with the Qubit protein assay. Aliquots of 20 µg protein were prepared and stored at -20 °C until use.

Soluble trypsin digestion kinetics The 8 M urea of the reconstitution buffer and the protein concentration were diluted to 1.6 M urea with 20 mM Tris-HCl pH 8.0/2 mM $CaCl_2$ and 1 mg/mL protein in a total volume of 20 µL. Soluble trypsin at a concentration of 0.1 mg/mL was added to a substrate/protease ratio 100:1 (100 ng) and incubated at 37 °C. Digestion was stopped by addition of 1/10 digestion volume of 10% (v/v) TFA, followed by dilution to 0.1 mg/mL protein with 0.1% (v/v) TFA in water for LC-MS/MS analysis or alternatively by addition of 10 µL SDS-PAGE sample buffer and boiling for 5 min before SDS-PAGE analysis (Fig. S3).

SPs-Try-AT digestion kinetics 20 µL of SPs-Try-AT were centrifuged for 10 s at 5000 g. The supernatant was carefully removed. An aliquot of 20 µg HEK cell lysate was diluted as described in the previous section and added to the pellets of SPs-Try-AT. Incubation at 37 °C was started by shaking in a thermomixer. After defined incubation times, the SPs-Try-AT beads were removed by fast centrifugation and the supernatant was transferred to a new aliquot. Samples were diluted to 0.1 mg/mL protein with 0.1% (v/v) TFA in water for LC-MS/MS analysis or alternatively by addition of 10 µL SDS-PAGE sample buffer and boiling for 5 min before SDS-PAGE analysis (Fig. S3).

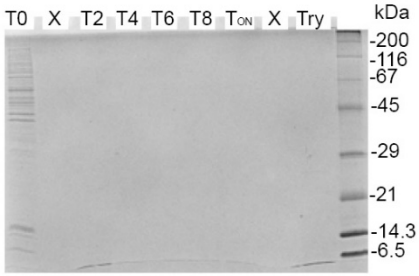


Fig. S 5 Coomassie stained gel of HEK cell lysates digested with trypsin. The annotation on top of gel stands for non-digested cell lysate (T0); empty lane (X); cell lysate digests with SPs-Try-AT at 37 °C for 2 h (T2), 4 h (T4), 6 h (T6), 8 h (T8) and overnight (ON), cell lysate digested for 6 h with soluble trypsin (Try) at 37 °C.

SDS-PAGE of tryptic HEK cell lysate digests 30 µL of digested cell lysates prepared for SDS-PAGE analysis were loaded on home-made 12.5% (T) SDS-PAGE. The gels were run at 75 V for 15 min followed by 45 min at 200 V. Proteins were then stained with Coomassie Blue.

LC-MS/MS analysis of tryptic HEK cell lysate digests Samples for LC-MS/MS analysis were diluted to 0.1 mg/mL protein with 0.1% (v/v) TFA in water, centrifuged for 1 min at 16000 g and 50 µL were transferred into an HPLC vial. Each sample was analysed three times by loading 5 µL onto a Pepmap100 Trap column by an EASY-nLC1000 UPLC system and separated by backflush onto the analytical column with a 60 min gradient from 5 to 40 % with 95 % acetonitrile/0.1% formic acid at a flow

rate of 350 nL/min. The effluent of the nanoLC column was electrosprayed directly into the mass spectrometer. Peptide precursor mass information in the m/z range of 360-1400 was acquired at resolution 70,000 with an automatic gain control target of $1e^6$ and a maximum injection time of 50 ms, followed by top10 peptide fragment spectra with resolution 17500, a target of $1e^5$ and a maximum injection time of 110 ms using a normalised HCD collision energy of 27% and applying a dynamic exclusion for 20s.
Fragment spectra interpretation was conducted with open source software MaxQuant and a transproteome pipeline setup on a remote server infrastructure. The option of match between run was disabled with MaxQuant software. Non-normalised intensity based absolute quantitation (iBAQ) and normalised label-free quantitation (LFQ) protein intensities were calculated by MaxQuant together with number of unique peptides, peptide spectrum matches and missed trypsin cleavage sites with both software tools. The database search was done against a recent Swissprot homo sapiens protein sequence database applying a strict trypsin cleavage rule (no cleavage when proline follows after arginine or lysine) allowing for up to four missed cleavages, variable modification of acetylation on the protein N-terminus and oxidation of methionine as well as a fixed carbamidomethyl modification of cysteines.

(1) Beerli, R. R.; Hell, T.; Merkel, A. S.; Grawunder, U. *PLoS One* **2015**, *10*, e0131177.

(2) Cumbo, A.; Lorber, B.; Corvini, P. F. X.; Meier, W.; Shahgaldian, P. *Nat. Commun.* **2013**, *4*, 1503.