Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Immobilisation and stabilisation of glycosylated enzymes on boronic acid-functionalised silica nanoparticles

Seyed Amirabbas Nazemi, Magdalena Olesińska, Cinzia Pezzella, Simona Varriale, Chi-Wei Lin, Philippe F.-X. Corvini and Patrick Shahgaldian

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Materials and methods

Chemicals - Tetraethyl orthosilicate (TEOS, ≥ 99%), (3-aminopropyl)triethoxysilane (APTES, ≥ 98%), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), dimethyl sulfoxide (anhydrous, ≥ 99.9%),Triton X-100 (molecular biology grade), ammonium bicarbonate (≥ 98%), sodium phosphate dibasic (ACS grade, anhydrous, ≥ 99%), potassium phosphate monobasic (ACS grade, ≥ 99%), peroxidase from horseradish (HRP, lyophilized powder), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, HPLC grade, ≥ 98%) were purchased from Sigma-Aldrich (Switzerland). Recombinant laccase A1b (POXA1b) was produced as previously described.[1] Citric acid (ACS grade, ≥ 99.5%) and bovine serum albumin (BSA, 98%) were purchased from Carl Roth (Germany). Spectrum Spectra/Por® 7 membrane tubing (50kDa MWCO) was purchased from Thermo Fisher Scientific. Bradford protein quantification kits were purchased from Bio-Rad. 4-Chloromethylphenylboronic acid (CMBA, 96%) was purchased from ABCR (Netherlands). 3-Formylphenylboronic acid (FBBA) was purchased from Alfa Aesar. Hydrogen peroxide solution (TraceSelect Ultra, ≥ 30%) was purchased from Fluka (Netherlands).

Buffer solutions - Buffer solutions were prepared with nanopure water. ABC buffer: 50 mM and 10 mM ammonium bicarbonate, pH 7.8. NaPB: 29 mM sodium phosphate dibasic, 21 mM sodium phosphate monobasic, pH 7.0. McIlvaine (MPC) buffer: 200 mM sodium phosphate dibasic, 100 mM citric acid, pH 3.6. KPB: 100 mM potassium phosphate monobasic, pH 5.0. HRP activity buffer: 40 mM potassium phosphate buffer, 0.25%(w/v) BSA, 0.5% (v/v) Triton X-100, pH 6.8.

Equipment - Activity assays and bioconjugation reactions were performed and monitored using a Synergy H1 spectrometer (BioTek, Switzerland) into a 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). Particles were imaged using a Zeiss SUPRA® 40VP scanning electron microscope (Germany) and a Bruker MultiMode® scanning probe microscope system (USA). Particle sizes were measured using ImageJ free software and processed with Nanoscope® software package. SNPs' surface charge (ζ-potential) was measured using dynamic light scatting (DLS; Zetasizer Nano S90, Malvern, UK). Digested enzyme samples were analysed on a calibrated Q Exactive® mass spectrometer (Thermo Fischer Scientific, Germany) coupled to a Waters Acquity UPLC M-Class system (Waters, USA).

Experimental procedures

Amino-modification of SNPs - Silica nanoparticles (SNPs) were produced following a method described by Cumbo et al.[2] inspired by the Stöber method.[3] The nanoparticles synthesised were diluted in 400 mL H_2O to obtain a solution of [SNPs] = 3.2 mg/mL. APTES (1.11 mmol) was added to the previous solution, and the reaction mixture was stirred at 20 °C, 400 rpm for 90 minutes. Subsequently, the suspension was centrifuged at 4000 rpm for 15 minutes, and the supernatant was removed. The particles were resuspended in nanopure H_2O (50 mL) and submitted to ultrasonic treatment. This washing step was repeated twice. The particles produced, SNP-NH_2 were then resuspended in water (50 mL) and stored at 4 °C.

Boronic acid grafting - To a suspension of SNP-NH_2 (50 mL, 3.2 mg/mL) was added CMBA (0.1 mmol, dissolved in DMSO). The reaction mixture was stirred at 65 °C, 500 rpm for 2 hours. The suspension was subsequently washed twice, sonicated and resuspended in 50 mM ABC buffer (5 mL) to yield SNP-BA (32 mg/mL). The ζ-potential of SNPs, SNP-NH_2, and SNP-BA was measured to verify each step modification in 100 mM NaPB pH 7.0. The summary of the three-run measurement of nanoparticles is presented in Figure S1.

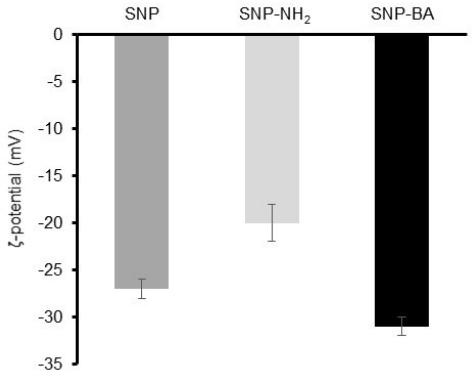

Fig. S1. ζ-potential of silica nanoparticles (SNP), amino-modified nanoparticles (SNP-NH_2), and boronic acid functionalised nanoparticles (SNP-BA)

Laccase immobilisation - To a suspension of SNP-BA (5 mL, 32 mg/mL) was added 5 mL (in ABC buffer) POXA1b (final concentration of 48 µg/mL) to reach [SNP-BA] = 16 mg/mL. The resulting mixture was stirred at 20 °C, 400 rpm for 1 h to produce SNP-BA-POXA1b. The nanoparticles were centrifuged at 700 rcf for 5 minutes. A Bradford assay was performed on the supernatant collected after enzyme immobilisation, which showed that 53% of the enzyme was immobilised at the surface of SNP-BA; *cf.* Table S1. As for controls, the control experiments for bare silica nanoparticles (SNPs) and amino-modified nanoparticles (SNP-NH_2) were conducted as previously described by replacing the SNP-BA particles with SNPs and SNP-NH_2. In the boronic acid negative control, pre-treated POXA1b (PT-POXA1b) with saturated binding sites (diols) was used. In 50 mM ABC buffer was dissolved 3-formylphenylboronic acid (FBBA, 0.5 mmol). To the FBBA-containing ABC buffer was added POXA1b and incubated for 1 h at 20 °C under 400 rpm stirring. The pre-treated POXA1b (final concentration of 55 µg/mL) was used for immobilisation reaction, as previously described above. A summary of the results is presented in Table S2.

Table S1. Protein quantification carried out on reaction supernatants after immobilisation reaction of POXA1b.

OD Supernatant	OD Blank	[POXA1b] initial (µg/mL)	[POXA1b] supernatant (µg/mL)	[POXA1b] immobilised (µg/mL)	Average [POXA1b] immobilised (µg/mL)	Average [POXA1b] immobilised (µg/mg SNP)
0.329	0.309	48	20	28		
0.326	0.305	48	21	27	25	1.56
0.330	0.303	48	27	21		

Table S2. Laccase immobilisation and enzymatic activity results summary. OD values are provided as an average of triplicate measurements.

Control	OD Supernatant	OD Blank	[POXA1b] initial (µg/mL)	[POXA1b] supernatant (µg/mL)	[POXA1b] immobilised (µg/mg SNP)	Enzymatic activity (U/mg SNP)
SNP-BA-POXA1b	**0.3283**	**0.3057**	**48**	**23**	**1.56**	**0.46**
SNP-BA-PT-POXA1b	0.3653	0.3150	55	50	0.31	0.10
SNPs-POXA1b	0.3528	0.3142	49	39	0.63	0.21
SNP-NH_2-POXA1b	0.3710	0.3227	54	48	0.34	0.02

HRP immobilisation - To SNP-BA (5 mL, 32 mg/mL) was added 5 mL (in ABC buffer) HRP (final concentration of 47 µg/mL) to reach [SNP-BA] = 16 mg/mL. The resulting mixture was stirred at 20 °C, 400 rpm for 1 h to produce SNP-BA-HRP. The resulting nanoparticles were centrifuged at 700 rcf for 5 minutes. A Bradford assay was performed on the supernatant collected after enzyme immobilisation which showed that 56% of the enzyme was immobilised at the surface of SNP-BA; *cf.* Table S3.

Table S3. Protein quantification carried out on reaction supernatants after immobilisation reaction of HRP.

OD Supernatant	OD Blank	[HRP] initial (µg/mL)	[HRP] supernatant (µg/mL)	[HRP] immobilised (µg/mL)	Average [HRP] immobilised (µg/mL)	Average [HRP] immobilised (µg/mg SNP)
0.3351	0.3173	47	16	31		
0.3401	0.3151	47	25	22	26	1.63
0.3366	0.3163	47	20	27		

Laccase activity assay - For POXA1b enzymatic activity measurement, ABTS oxidation assay was carried out following the method described by Zimmermann et al.[4] where enzymatic activity is determined by monitoring the oxidation rate of the substrate ABTS to its stable, blue-green radical cation ($ABTS^{\bullet+}$) at 420 nm (molar extinction coefficient ε_{420} = 30800 $M^{-1}cm^{-1}$) for 420s (8s intervals) with 96-well microplates using a microplate reader. One unit (U) was defined as the amount of enzyme oxidising 1 µmol of ABTS per minute. In a typical experiment, POXA1b-containing samples (soluble, immobilised, and shielded POXA1b) were diluted in MPC buffer (pH 3.6) and added to a 96-well microplate and monitored immediately after the addition of 150 µL of 2 mM ABTS solution

(also in MPC buffer). The specific activity of the soluble POXA1b was calculated to be 299 U/mg enzyme (Table S4).

HRP activity assay - The enzymatic activity of the HRP-containing samples (soluble, immobilised, and shielded HRP) was determined by following ABTS oxidation as substrate at 405 nm (molar extinction coefficient ε_{405} = 36800 $M^{-1}cm^{-1}$). To a solution of ABTS (2.9 mL, 9.1 mM, in 100 mM KPB buffer, pH 5) was added HRP-containing sample (0.05 mL, diluted in HRP activity buffer). The mixture was equilibrated by mixing at room temperature for 5 minutes. Hydrogen peroxide (H_2O_2, 0.1 mL, 0.3% (w/w)) was added to the samples and mixed by inversion. Kinetics were monitored in a 96-well microplate by recording the increase in absorbance at 405 nm for 4 minutes. The specific activity of the soluble HRP was calculated to be 1245 U/mg enzyme. A summary of the results of both activity assays (POXA1b and HRP) is presented in Table S4.

Table S4. POXA1b and HRP immobilisation and enzymatic activity results summary

Enzyme	Initial concentration (µg/mL)	Immobilisation (µg/mg SNP)	Immobilisation yield (%)	Soluble enzyme activity (U/mg enzyme)	Immobilised enzyme activity (U/mg enzyme)	Activity retention (%)
POXA1b	48	1.56	53	299	295	99
HRP	47	1.63	56	1245	1165	94

Laccase and HRP shielding - SNP-BA-POXA1b and SNP-BA-HRP (10 mL, 3.2 mg/mL) were incubated with TEOS (0.134 mmol) in 10 mM ABC buffer at 10 °C, 400 rpm for 1 h. Subsequently, APTES (0.026 mmol) was added to the reaction media. The resulting mixture was stirred at 10 °C, 400 rpm for 60 minutes. Aliquots of 2 mL were collected every 15 minutes. The resulting nanoparticles were centrifuged at 700 rcf for 5 minutes, washed and resuspended in 0.4 mL ABC buffer ([SNP-BA-POXA1b] and [SNP-BA-HRP] = 16 mg/mL). The resulting suspension were allowed to cure at 20 °C for 18 hours and finally stored at 4 °C.

Scanning electron microscopy - A solution of bare SNPs, shielded SNP-BA-POXA1b and shielded SNP-BA-HRP in nanopure water was prepared and spread on a silicon substrate. The samples were allowed to dry at room temperature under atmospheric conditions and were subsequently sputter-coated with gold for 15 seconds 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 ImageJ free software. 100 measurements, at least, were performed for each sample (Table S5 and S6). Statistical analysis of SEM micrographs on shielded SNP-BA-POXA1b is shown in Figure S2 and the kinetics (layer growth and activity retention) of HRP are presented in Figure S3.

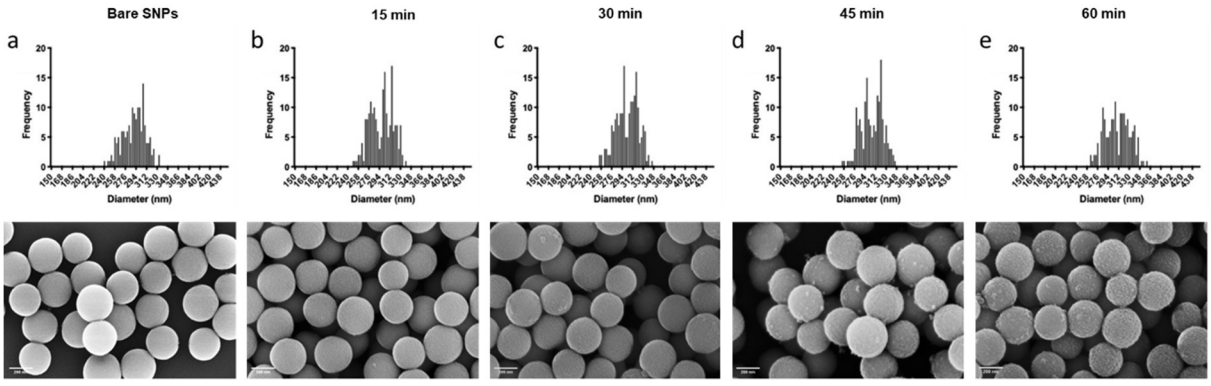

Fig. S2. Statistical analysis of SEM micrographs of bare SNPs (a) and SNP-BA-POXA1b after 15 (b), 30 (c), 45 (d) and 60 (e) minutes of reaction with APTES and TEOS. Scale bars represent 200 nm.

Table S5. Layer growth kinetics of SNP-BA-POXA1b results summary

Reaction time (min)	0	15	30	45	60
Mean diameter (nm)	290	292 ± 0.4	300 ± 0.6	306 ± 0.4	310 ± 0.6
Layer thickness (nm)	0	1	5	8	10

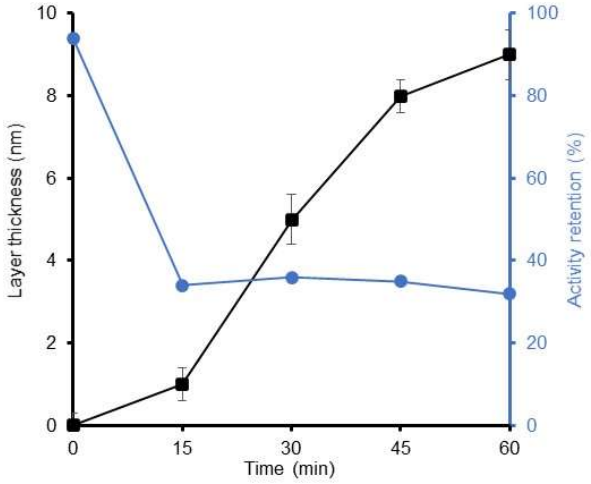

Fig. S3 Kinetics of layer growth (black squares) and activity retention (blue circles) on HRP enzyme of each timepoint measured on SEM micrographs; each point is the average of at least 100 SNPs; error bars represent ± 0.5 nm.

Table S6. Layer growth kinetics of SNP-BA-HRP results summary

Reaction time (min)	0	15	30	45	60
Mean diameter (nm)	290	292 ± 0.5	302 ± 0.15	306 ± 0.5	308 ± 0.5
Layer thickness (nm)	0	1	6	8	9

Atomic force microscopy - A solution of bare SNPs and shielded SNP-BA-POXA1b in nanopure water was prepared and placed onto freshly cleaved mica sheet. The samples were dried at 25 °C for 24 hours. AFM micrographs were acquired using tapping mode with a rectangular cantilever (RTESPA-150; tip radius = 8 nm, f = 150 kHz, k = 5 N/m, Bruker, USA). The image processing (flattening and height adjustment) and analysis (roughness values) of AFM micrographs were performed using the Nanoscope and Gwyddion software package. Surface roughness parameters were examined on five different positions for each sample.

Laccase glycan profile analysis - 20 µg of POXA1b was reduced with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate buffer pH 8.5 for 1 hour at 37 °C and subsequently alkylated with 20 mM iodoacetamide (IAA) for 1 hour at 37 °C in the dark. The sample was digested with 1 µg of sequencing-grade modified trypsin (Promega) and incubated for 16 hours at 37 °C. Half of the sample was further digested by GluC endopeptidase (Roche). Both samples were dried and dissolved in 2.5% acetonitrile with 0.1% formic acid. Both tryptic and trypsin/GluC Samples were analysed on a calibrated Q Exactive® mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to a Waters Acquity UPLC M-Class system (Waters, Milford, USA) with a Picoview® nanospray source 500 model. The tryptic samples were dissolved in 2.5 % acetonitrile with 0.1 % formic acid, loaded onto an Acclaim PepMap 100 trap column (75 µm × 20 mm, 100 Å, 3 µm particle size) and separated on a nanoACQUITY UPLC BEH130 C18 column (75 µm × 250 mm, 130 Å, 1.7 µm particle size), at a constant flow rate of 300 nL/min, with a column temperature of 50 °C and a linear gradient of 1 − 35 % acetonitrile/0.1 % formic acid in 42 minutes, followed by a sharp increase to 98 % acetonitrile in 2 minutes and then held isocratically for another 10 minutes. For DDA (the site-occupancy) analysis, one scan cycle comprised a full-scan MS survey spectrum, followed by up to 12 sequential HCD scans based on the intensity. For glycosylation profiling analysis, full-scan MS spectra (600 – 2000 m/z) were acquired in the FT-Orbitrap at a resolution of 70,000 at 400 m/z, while HCD

MS/MS spectra were recorded in the FT-Orbitrap at a resolution of 35,000 at 400 m/z. HCD MS/MS spectra were performed with a target value of 5e5 by the collision energy setup at normalised collision energy of 25. The identification of each glycoform was first done by Byonic 3.11 software (Protein Metrics) and manually confirmed by its corresponding MS/MS spectrum.[5] The list of masses of all glycoforms detected and analysed can be found in Table S7. The summary of the glycosylation profile on POXA1b is presented in Figure S4. There are six putative N-glycosylation sites on POXA1b (Figure S4a). The observed glycan structures on each site are depicted. The MS profile at N454 is shown in Figure S4b. Based on the observed m/z Man8-13 structures could be observed on this site. The MS/MS spectrum (Figure S4c) showed the fragment ions from the peptide backbone containing N454 site and Y1 ion, peptide with HexNAc, which confirmed the identity of this glycopeptide with Man9 structure.

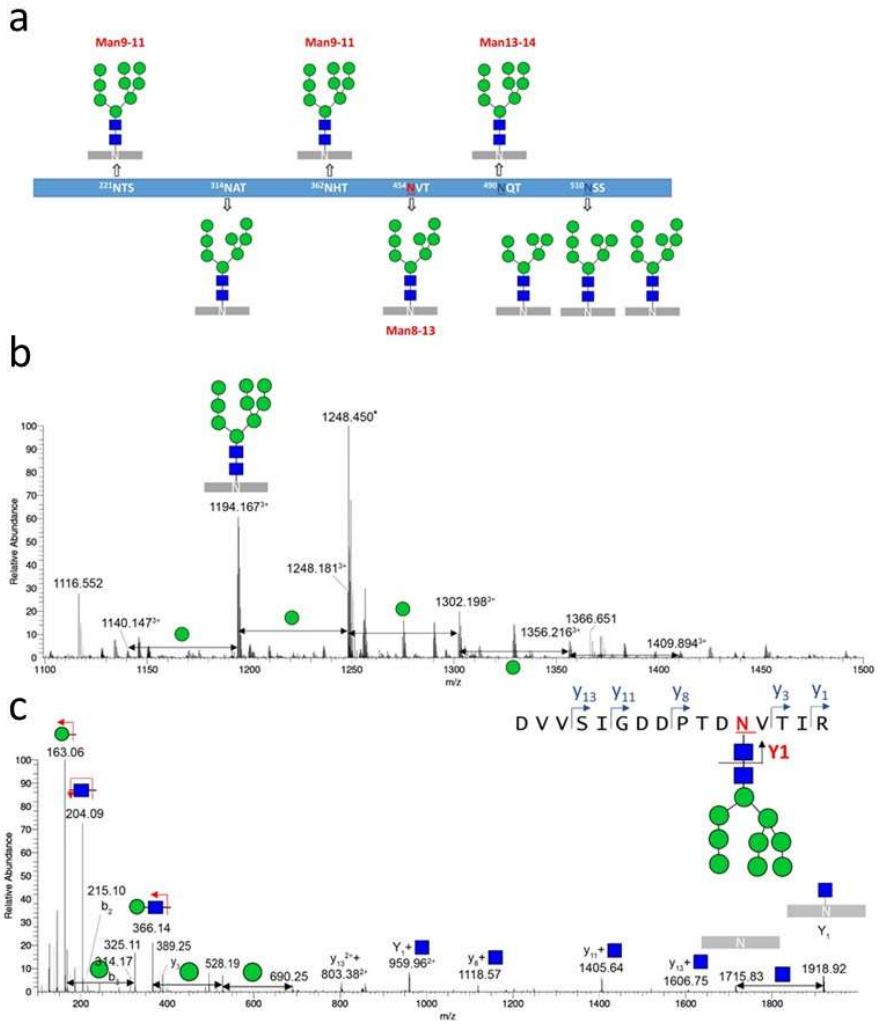

Figure S4. Summary of glycosylation profile on POXA1b. The observed glycan structures on each site are presented (a). The MS profile at N454 is depicted (b), and the MS/MS of 1194.1646 (+3) is shown in detail (c). The nomenclature of peptide fragment ions and glycan fragmentation ions was previously described.[6,7,8]

Table S7. Observed glycopeptides from POXA1b results summary

Site	sequence	m/z	Structure[1]	Score[2]
221	R.LI**N**TSCDSNYQFSI.D[3]	1119.4392^{3+}	Man9	30[4]
		1173.4578^{3+}	Man10	100.5
		1127.4745^{3+}	Man11	30[4]
314	S.IDPTTPEQ**N**ATNP.L[3]	1001.7345^{3+}	Man8	83.5
362	D.PATALFTAN**N**HTFVP.P[3]	1155.8244^{3+}	Man9	124.2
		1209.8356^{3+}	Man10	184.2
		1263.8502^{3+}	Man11	139.3
454	R.DVVSIGDDPTD**N**VTIR.F[5]	1140.1474^{3+}	Man8	436.9
		1194.1646^{3+}	Man9	573.0
		1871.7651^{2+}	Man10	407.8
		1302.2005^{3+}	Man11	754.8
		1356.2170^{3+}	Man12	458.0
		1409.8940^{3+}	Man13	366.6
490	E.GV**N**QTAAANPVP.E[3]	1217.8181^{3+}	Man13	30[4]
		1271.8404^{3+}	Man14	30[4]
510	E.AWNNLCPIY**N**S.S[3]	891.6981^{3+}	Man6	30[4]
		945.7169^{3+}	Man7	30[4]
		999.7324^{3+}	Man8	30[4]

Notes: [1]ManX structure represents high mannose *N*-glycan structure, including GlcNAc2ManX (X from 6-13); [2]the score was obtained from Byonic results; [3]Only observed in the sample treated with trypsin and GluC endopeptidase; [4]Although the score was low, the spectra could provide sufficient fragment ions for its corresponding assignment, the data were inspected manually; [5]the peptides were observed in both tryptic and trypsin/GluC samples.

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