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Supporting Information

Biocatalytic Atom Transfer Radical Polymerization in a Protein Cage Nanoreactor

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1. Experimental Section

1.1. Materials

Chemicals were obtained from Sigma-Aldrich and used without further purification unless otherwise noted. ^{1}H-NMR solvents were obtained from Cambridge Isotope Laboratories. The linkers maleimido trioxa-6-formyl benzamide (MTFB) and succinimidyl 6-hydrazinonicotinate (HyNic) were purchased from SoluLink (San Diego, USA). Buffers were produced using double-distilled water. Poly(ethylene glycol) methylether acrylate (PEGA) with a number average molecular weight of 480 g mol^{-1} was filtered over a neutral aluminium oxide column. 2-Hydroxyethyl-2-bromoisobutyrate (HEBIB) was purified by vacuum distillation. Tetrahydrofurane (THF; inhibitor-free for HPLC) was purchased from Sigma-Aldrich. A mutant of thermosome (THS) from the archaea *Thermoplasma acidophilum* with three point mutations (C363A in the α-subunit and K316C and C364A in the β-subunit) was expressed in *E. coli* as described previously.[1] Horseradish peroxidase (HRP; highly stabilized, essential salt-free, lyophilized powder with an activity of 200-300 units per mg solid (using pyrogallol)) was purchased from Sigma-Aldrich. RNase A from bovine pancreas was obtained from Roche Diagnostics.

1.2. Methods

The M_n and the $Đ$ of the polymers were determined by gel permeation chromatography (GPC). Samples for GPC (0.1 ml) were removed from the reaction mixture without separation of the protein and filtered over a small plug of basic aluminum oxide in order to remove salts and protein. The plug was washed with additional 0.2 ml of water and the filtrates were combined. For ^{1}H-NMR spectroscopy, the filtrate was diluted with D_2O and analyzed with a Bruker DPX-NMR (400 MHz) instrument at room temperature. Conversion of PEGA was calculated from the baseline corrected spectra using the integrals of the signals between 6.00 ppm and 6.45 ppm, which correspond to three protons of the residual monomer, and the overlapping signals at 3.38 ppm, which correspond to the three O-CH_3 endgroup protons of the monomer and polymer side chains. For GPC, the sample was lyophilized and redissolved in the GPC eluent to concentrations of 1 mg ml^{-1}. GPC measurements were performed on a SECcurity GPC system (PSS, Mainz) using a refractive index detector, with Agilent PLgel columns (3 µm guard + 3 x 5 µm mixed-C) thermostatted to 40°C and THF as mobile phase at a flow rate of 1 ml min^{-1}. Calibration was carried out with narrow polystyrene standards (PSS, Mainz). GPC curves were smoothed and plotted using Igor Pro (WaveMetrics, Inc., Lake Oswego, OR, USA). Small angle X-ray scattering (SAXS) experiments were performed with a Bruker AXS Metaljet Nanostar. The X-ray are generated by focusing electrons onto a micro-size liquid metal jet of an alloy consisting of 95% Ga and 5% In. This setup allows for a power load of > 100 kW mm^2. The scattering patterns are detected by a Vantec 2000 2D detector. Exposure times were 3600 s. About 20 µl of sample with a THS concentration of 1.5 mg ml^{-1} was loaded into a glass capillary (d = 1 mm, thickness of the wall = 0.01 mm), the capillary was closed by melting the top of the capillary, the closed capillary was put into the Nanostar, vacuum was applied and the measurement was started. The data were azimuthally averaged with SAXS v.4.1.36 Bruker software and fitted with BrukerAXS Nanofit software (version 1.2.1.1). UV/Vis analyses were performed on a SpectraMax M5^e (Molecular Devices, LLC, CA, USA) with 1 cm quartz

cuvettes (Hellma, Germany) and on a NanoDrop (Thermo Scientific). Protein concentrations were determined from the absorption at 280 nm, using an extinction coefficient of 210 880 M^{-1} cm^{-1} for the hexadecameric THS complex,[1] 95 000 M^{-1} cm^{-1} at 280 nm for HRP,[2] 102 000 M^{-1} cm^{-1} at 403 nm for HRP[3] and 248 880 M^{-1} cm^{-1} at 280 nm for the THS-HRP conjugate with 0.4 bound HRP. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with self-casted 12% polyacrylamide gels and tris-HCl as running buffer in a Biorad Miniprotean 3 cell setup at 200 V for 70 min. Novex BenchMark Protein Ladder (Invitrogen) was used as molecular weight marker. Gels were Coomassie stained with SimplyBlue SafeStain (Invitrogen) and scanned on a Gel Doc EZ imager (Biorad). For transmission electron microscopy (TEM), THS-HRP samples (5 µl of 20 nM solution) were deposited on glow-discharged grids for 60s, washed twice with ddH_20, and subsequently negatively stained twice with 1% uranyl acetate solution. A FEI Morgagni 268 D transmission electron microscope at an accelarating voltage of 80 kV was used to image the samples. Graphical images of HRP (PDB: 2ATJ)[4] and THS (As no crystal structure of the THS in its open conformation is available in the databases, structural data from TRiC/CCT was used to illustrate a chaperonin in its open conformation (PDB: 4A0O;[5] this structure is in compliance with the structural data of THS[6]) were produced with the UCSF Chimera package[7] from the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco.

1.3. THS-MTFB Modification

8.1 µl of 35.7 mM MTFB in DMSO was diluted in 41.9 µl modification buffer (100 mM NaH_2PO_4, 150 mM NaCl, 1 mg ml^{-1} phenyl methyl sulfonyl fluoride (PMSF), pH 6.5), which lead to 50 µl 5.78 mM MTFB. 100 µl of 10 µM THS solution was added, resulting in a final volume of 150 µl and final concentrations of 6.4 µM THS and 1.93 mM MTFB (> 25 fold excess of MTFB to cysteins in THS). The solution was incubated under gentle shaking at 37°C

for 2.5 h. Free MTFB was removed from the THS-MTFB conjugate by four cycles of size exclusion spin centrifugation at 13000 rpm using diafiltration devices (Amicon Ultra 0.5 ml, 100 kDa molecular weight cut-off (MWCO); Millipore, MA, USA) in a MiniSpin centrifuge (Eppendorf, Switzerland). By refilling the diafiltration devices every cycle with conjugation buffer (100 mM NaH_2PO_4, 150 mM NaCl, 1 mg ml^{-1} PMSF, pH 6.0), we changed the buffer system for the next reaction.

1.4. HRP-HyNic Modification

HyNic solution: 20 µl of 100 mM HyNic in DMSO were added to 80 µl modification buffer, yielding 100 µl of 20 mM HyNic. HRP solution: 1.76 mg of HRP were dissolved in 100 µl of modification buffer, yielding 100 µl of 400 µM HRP. 100 µl of the HRP solution were added to 30 µl modification buffer and 20 µl of the HyNic solution (150 µl, 266.67 µM HRP, 2.67 mM HyNic, 10 fold excess of HyNic) and shaken for 1.5 h at room temperature. Unreacted HyNic was removed from the HRP-HyNic conjugate by four cycles of size exclusion spin centrifugation at 13000 rpm using 30k MWCO diafiltration devices (Amicon Ultra 0.5 ml, Millipore, MA, USA) in a MiniSpin centrifuge. By refilling the diafiltration devices every cycle with conjugation buffer, we changed the buffer system for the next reaction.

1.5. RNase-HyNic Modification

1.24 mg HyNic were dissolved in 124 µl DMSO. RNase was dissovled in modification buffer to a concentration of 8 mg ml^{-1}. 625 µl of the RNase A solution were added to 4269 µl modification buffer and 106 µl of the HyNic solution (final solution: 5 ml, 73 µM RNase, 730 µM HyNic, 10 fold excess of HyNic) and shaken for 2.5 h at room temperature. Unreacted HyNic was removed from the RNase-HyNic conjugate and the buffer was exchanged to the conjugation buffer by means of a HiTrap desalting column (Sephadex G-25; G.E. Healthcare). The final sample volume was 7 ml.

1.6. THS-HRP Conjugation

200 µl THS-MTFB solution and 200 µl HRP-HyNic solution were mixed and incubated at room temperature under shaking for 14 h. Unbound HRP was separated from THS-HRP with a HiPrep 16/60 Sephacryl S-200 high resolution size exclusion column (GE Healthcare Life Science, Buckinghamshire, UK) in column buffer (100 mM NaH_2PO_4, 150 mM NaCl, 1 mg ml^{-1} PMSF, pH 7.4) using a ÄKTAprime plus fast protein liquid chromatography (FPLC) system (GE Healthcare Life Science) with two UV/Vis detection chanels at 280 nm and 403 nm (Figure S3). The THS-HRP fraction was collected, filtered through a Millex-GV 0.22 µm syringe filter with PVDF membrane (Millipore, MA, USA) and concentrated using 100 kDa MWCO diaflitration devices (Amicon Ultra 5 ml, Millipore, MA, USA) at 3750 rpm to a final THS-HRP concentration of 5 mg ml^{-1} (equals 4.9 mg ml^{-1} THS) with a ratio HRP:THS of 0.4 (concentrations determined by UV/Vis spectroscopy). Aliquotes of this stock solution were used for UV/Vis, SAXS, TEM and SDS-PAGE to further characterization of the THS-HRP conjugate. THS-HRP for polymerization was washed four times with doubly distilled water (ddH_2O) in 100 kDa MWCO diafiltration device remove residual buffer salts and aliquoted to 2.6 mg THS-HRP containing approx. 50 µg HRP in 1050 µl ddH_2O.

To confirm a successful purification of THS-HRP, a SDS-PAGE was performed in comparison to unmodified THS and free HRP (see Figure S1). UV/Vis of THS-HRP was used to calculate the average number of HRP molecules per THS using the absorption at 403 nm (HRP) and at 280 nm (overlay signal of THS and HRP). Further, the number of bisaryl hydrazone linker groups formed per THS and HRP were determined from the absorbance at 354 nm using an extinction coefficient $\varepsilon(\text{hydrazone})_{354\ \text{nm}} = 29000\ M^{-1}\ cm^{-1}$.[8,9]

1.7. THS-RNase Conjugation

69.4 µl THS-MTFB solution (14.4 mg ml^{-1}) and 333 µl RNase-HyNic solution were mixed and incubated at room temperature under shaking for 16 h. Unbound RNase was separated from

THS-RNase by size exclusion chromatography in anaology to the THS-HRP purification. After concentration of the combined THS-RNase fractions in an Amico Ultra-15 (100 kDa MWCO) diafiltration device, 400 µl of a 1.31 µM THS-RNase (equals 1.83 mg ml^{-1} THS) with a ratio RNase:THS of 0.99 were obtained (concentrations determined by UV/Vis spectroscopy).

1.8. ARGET ATRP of PEGA with THS-HRP

0.1 ml PEGA (220 µmol, 66.7 equiv.) and 0.7 mg 2-hydroxyethyl-2-bromoisobutyrate (HEBIB) (3.3 µmol, 1.0 equiv.) were dissolved in 300 µl of a ddH_2O/THF (7:3 *v*/*v*) mixture. To the aliquoted 1050 µl of THS-HRP, 450 µl of THF was added. The resulting 1500 µl THS-HRP (2.6 mg, 2.8 nmol, 8.5 10^{-4} equiv.) containing approx. 50 µg HRP (1.1 nmol, 3.4 10^{-4} equiv.) in a ddH_2O/THF (7:3 *v*/*v*) mixture was added to the monomer/initator solution and argon was bubbled through the solution for 20 min. The polymerizations were started by adding 100 µl of 3.15 mM sodium ascorbate solution (0.75 mg, 3.75 µmol, 0.12 equiv.) in a ddH_2O/THF (7:3 *v*/*v*) mixture that had been bubbled thoroughly with argon for 20 min. The reaction was stirred for 20 h and stopped by adding 550 µl non-deoxygenated ddH_2O and exposure to ambient air.

1.9. ARGET ATRP of PEGA with free HRP

0.1 ml PEGA (220 µmol, 66.7 equiv.) and 0.7 mg HEBIB (3.3 µmol, 1.0 equiv.) were dissolved in 300 µl of a ddH_2O/THF (7:3 *v*/*v*) mixture. 1500 µl of free HRP (50 µg, 1.1 nmol, 3.4 10^{-4} equiv.) in a ddH_2O/THF (7:3 *v*/*v*) mixture was added and argon was bubbled through the solution for 20 min. The polymerizations were started by adding 100 µl of 3.15 mM sodium ascorbate solution (0.75 mg, 3.75 µmol, 0.12 equiv.) in a ddH_2O/THF (7:3 *v*/*v*) mixture that had been bubbled thoroughly with argon for 20 min. The reaction was stirred for 20 h and stopped by adding 550 µl non-deoxygenated ddH_2O and exposure to ambient air.

2. Supporting Data

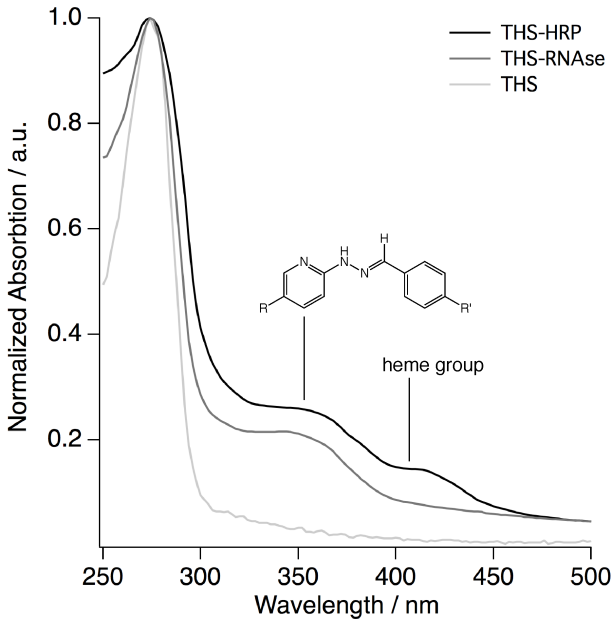

Figure S1. UV/Vis spectra of THS-HRP conjugate, THS-RNase conjugate and THS. The bisaryl hydrazone bond at 354 nm is only present if an enzyme is encapsulated into the THS and the Soret band of HRP at about 400 nm is only visible when HRP is encapsulated.

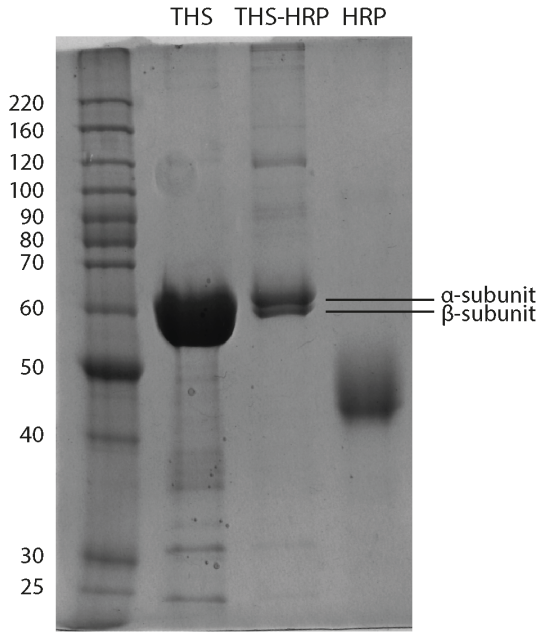

Figure S2. SDS-PAGE of THS, THS-HRP and HRP. The absence of a band of free HRP in the THS-HRP conjugate indicates that the excess of enzyme was quantitatively removed from the conjugate.

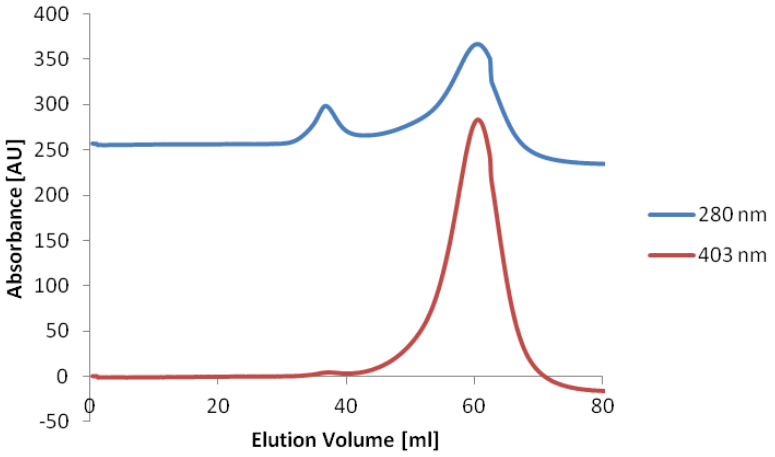

Figure S3 FPLC elution chromatogram of the purification of THS-HRP after the conjugation of THS with HRP. HRP has, beside the protein specific absorption at 280 nm, an additional absorption band at 403 nm, which is specific for the heme group. The first peak in the 280 nm channel is accompanied by a a relative weak peak in the 403 nm channel. This fraction corresponds to the THS-HRP conjugate. It elutes earlier than free HRP due to its lager size. The later peak in the 280 nm as well as in the 403 nm channel at an elution volume of approx. 60 ml corresponds to free HRP. This peak is more intense than the THS-HRP peak because an excess of HRP over THS was used in the conjugation reaction to achieve maximum loading of THS.

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