Supporting Information for

Enzyme-mediated polymerization inside engineered protein cages

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Experimental

Materials. All chemical and biological reagents were purchased from commercial sources and used without further purification. LB medium was purchased from MP Biomedicals, LLC (California, USA.). Hemin chloride and 30% hydrogen peroxide were purchased from Merck Millipore (Darmstadt, Germany), δ-aminolevulinic acid from Acros Organics (Geel, Belgium), guaiacol, and lysozyme from Sigma-Aldrich (Missouri, USA), 3,3-diaminobenzidine (DAB) from Axon Lab AG (Baden, Switzerland), DNase I from Roche Diagnosis GmbH (Mannheim, Germany). Phusion high-fidelity DNA polymerase, T4 DNA ligase and all restriction enzymes used in this study were purchased from New England BioLabs (Massachusetts, USA). Oligonucleotides were synthesized by Microsynth AG (Balgach, Switzerland). The plasmid pcDNA3 APEX2-NES was a kind gift from Prof. Alice Ting (Addgene plasmid # 49386).

Physical and Biophysical measurements. UV-vis absorption spectra were acquired on a Lamda 35 spectrophotometer (Perkin-Elmer, Massachusetts, USA). Fast protein liquid chromatography (FPLC) was carried out on an NGC Quest 10 plus chromatography system (Bio-rad laboratories, California, USA) equipped with a multi-wavelength detector and BioFrac fraction collector. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem electrophoresis apparatus using precast PhastGel homogeneous 20 (GE healthcare Life Sciences, Chicago, USA). Steady-state stopped-flow experiments were performed on an Applied Photophysics SX18 stopped-flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a xenon arc lamp and a 1-cm path length. Transmission electron microscope (TEM) images were acquired on a Morgani 268 microscope (FEI, Oregon, USA) operated at 100kV. Dynamic light scattering measurements were performed on a Zetasizer Nano (Malvern, Worcestershire, UK).

Construction of pACYC_His6-GFP(+36)-APEX2. A plasmid encoding GFP(+36)-APEX2 was constructed in three steps from the previously described pACYC_His6-GFP(+36)-GGS plasmid.[1] The gene encoding maltose binding protein (MBP) was obtained from pQE-MBP-SRJ[2] using the primer pair MBP-F and MBP-R (Table 1). The resulting DNA fragment was digested by XhoI and SpeI and inserted into the corresponding digestion site in pACYC_His6-GFP(+36)-GGS, yielding pACYC_His6-GFP(+36)-GGS-MBP. To remove the sequence between *gfp(+36)* and $(ggs)_5$ that encodes the peptide SGGSMALER, overlap-extension PCR was carried out. Briefly, two fragments corresponding to the N and C

termini were synthesized with the primer pairs pAKZ3-for/pACYC-KpnI-rev and pACYC-KpnI-for/MBP-R (Table 1). The two PCR products were purified and then combined in an equimolar ratio for final PCR. The full length fragment was assembled using the primer pair pAKZ3-for and MBP-R. The fragment was digested by NdeI and SpeI and ligated into pACYC_His6-GFP(+36)-GGS-MBP, which had been digested with the same restriction enzymes, to give pACYC_His6-GFP(+36)-MBP. The gene encoding APEX2 was obtained from pcDNA3 APEX2-NES (Addgene plasmid # 49386)[3] using the primer pair APEX2-XhoI-for and APEX2-SpeI-rev. The resulting DNA fragment was digested by XhoI and SpeI and inserted into the same digestion site of pACYC_His6-GFP(+36)-MBP, yielding pACYC_His6-GFP(+36)-APEX2. DNA sequence was confirmed by the DNA sequencing service provided by Microsynth AG.

Expression and purification of GFP(+36)-APEX2. Plasmid pACYC_His6-GFP(+36)-APEX2 was transformed into the *E. coli* strain BL21(DE3) for protein expression. A single colony of freshly transformed cells was cultured overnight in 10 mL of LB medium containing 34 μg/mL chloramphenicol. The culture was used to inoculate 1 L of LB medium which was incubated for ~2 h at 37 ºC and a shaking speed of 230 rpm. When the OD_{600} of the culture reached ~0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM to induce overexpression of the GFP(+36)-APEX2 gene. The induced culture was incubated for ~40 h at 18 ºC at the same shaking speed, and the cells were subsequently harvested by centrifugation at 5,500 *g* for 15 min. The pelleted bacterial cells were suspended in 20 mM Tris-HCl, 2 M NaCl at pH 7.4 (buffer A) with 10 mM imidazole containing 0.5% lysozyme and 0.01% DNase and sonicated to disrupt the cells. The lysate was centrifuged at 11,500 *g* for 15 min and the supernatant was subjected to affinity chromatography using Ni-NTA Agarose (Qiagen, Helden, Germany). His-tagged GFP(+36)-APEX2 was eluted with buffer A containing 300 mM imidazole. To reach maximum heme loading, the GFP(+36)-APEX2 was reconstituted with hemin chloride. Briefly, Ni-NTA purified GFP(+36)-APEX2 solution was rapidly mixed with two equivalents of hemin chloride dissolved in 10 mM NaOH, and incubated at room temperature for 1h. The GFP(+36)-APEX2 solution was then subjected to size-exclusion (SEC) chromatography on Superdex 200 10/300 GL (GE healthcare Life Sciences) at a flow rate of 0.5 ml/min using an NGC Quest 10 plus FPLC system equilibrated with buffer A. The fractions were monitored at 260, 280, 408, and 488 nm, collected using a BioFrac fraction collector, and analyzed by SDS-PAGE. If necessary, the protein solution was concentrated in an Amicon

Ultra-15 10-kDa cut-off centrifugal filter device (Merck Millipore, Massachusetts, USA). For long-term storage, glycerol (20% v/v) was added and the protein solution frozen at -80 °C.

Protein concentration was determined spectroscopically using ε_{280} = 34,840 M^{-1} cm^{-1} and ε_{488} = 36,600 M^{-1} cm^{-1} (Fig. S1).[1] The purified GFP(+36)-APEX2 samples had A_{405}/A_{280} ratios ≈ 2.0. After subtracting the contribution of GFP(+36) from the A_{280} value obtained for the fusion protein using an ε_{280} of 18,910 M^{-1} cm^{-1} for GFP(+36), a A_{405}/A_{280} ratio >4 was calculated for the heme protein, consistent with full heme occupancy according to previous reports for APEX.[3] However, direct determination of heme content using a published heme pyridine chromogen assay[4] indicates that the heme occupancy of purified GFP(+36)-APEX2 is approximately 40%. In the main text, the number of active enzymes loaded into the capsids is thus 40% of the total number of cargo molecules.

Expression and purification of AaLS-13 and AaLS-neg. Capsid proteins AaLS-13 and AaLS-neg were expressed and purified as described before with slight modifications.[1,5] Briefly, a single colony of BL21 gold (DE3) transformed with the plasmid encoding AaLS-13 or AaLS-neg was used to inoculate an overnight culture (LB-medium supplemented with 150 µg/mL ampicillin). After ca. 16 h incubation at 30 °C, 5 mL of the culture was used to inoculate 500 mL LB medium (supplemented with 100 µg/mL ampicillin). The cells were grown at 37 °C shaking at 230 rpm until the OD_{600} reached 0.6-0.7, upon which expression was induced by adding 0.25 mM IPTG. After ca. 18 h at 25 °C, cells were harvested by centrifugation at 4,200 g and 4 °C for 15 min. Cell pellets were stored at -20 °C. For protein purification, cells were thawed on ice and incubated with 10 mL buffer B (50 mM Tris-HCl, 300 mM NaCl at pH 8.0) and 10 mM imidazole, 1 mg/mL lysozyme and 0.1 µL/mL benzonase (Sigma) for 30 min. Cells were lysed by sonication followed by centrifugation at 21,000 g at 25 °C for 45 min. The resulting supernatant was loaded onto 3 mL of Ni(II)-NTA agarose resin equilibrated with lysis buffer, washed with ca. 100 mL of buffer B containing 20 mM imidazole and eluted with the same buffer containing 500 mM imidazole. The buffer was changed to AaLS storage buffer (50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 5 mM EDTA) by repeated concentration and dilution using an Amicon Ultra-15 centrifugal filter unit (30 kDa MWCO) (Merck Millipore). A concentrated NaCl solution was added to the concentrated protein solution (1-1.5 mM) to give a final concentration of 600 mM. After at least 5 days of incubation at rt, AaLS-13/neg capsids were separated from assembly intermediates and pentameric capsid building blocks by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-400 HR

column (GE healthcare) and the AaLS storage buffer as the running buffer. Purified AaLS capsid could be stored for several months at rt.

Encapsulation of GFP(+36)-APEX2 in empty AaLS-capsids. The purified AaLS capsids (monomer concentration ~50 μM) were mixed with GFP(+36)-APEX2 in a capsid monomer to GFP(+36)-APEX2 ratio of 10:1, 25:1, etc. in 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and 1 mM EDTA. The mixture was analyzed by SEC on Superose 6 10/300 GL (GE healthcare Life Sciences) equilibrated with the same buffer. Negative-stain TEM experiments were carried out as reported previously.[6] Briefly, protein solutions (typical capsid monomer concentrations of ~5 μM) were deposited on glow discharged copper grids with carbon support film (01814-F, Ted Pella Inc., California, USA), washed twice with the buffer solution, and stained with 2% uranyl acetate prior to TEM analysis. The concentration of encapsulated GFP(+36)-APEX2 was determined by UV-vis spectroscopy. To account for considerable light scattering due to the capsid particles, background was subtracted from the spectrum using the UV-Vis-IR Spectral Software a|e (FluorTools.com, http://www.fluortools.com/software/ae) as described previously.[1] The background-corrected absorbance values were used to determine the concentration of encapsulated GFP(+36)-APEX2 and AaLS monomer using equations (1) and (2), respectively.

$$[\text{G-APEX}] = A_{488} / \varepsilon_{488}(\text{G-APEX}) \quad (1)$$

$$[\text{AaLS-monomer}] = (A_{280} - [\text{G-APEX}] \times \varepsilon_{280}(\text{G-APEX})) / \varepsilon_{280}(\text{AaLS}) \quad (2)$$

where A_{488} and A_{280} are the background-corrected absorbance values measured for AaLS/GFP(+36)-APEX2 complexes and ε_{488}(G-APEX) = 36,600 M^{-1} cm^{-1}, ε_{280}(G-APEX) = 34,840 M^{-1} cm^{-1} and ε_{280}(AaLS) = 13,980 M^{-1} cm^{-1}. To determine the number of enzymes per capsid, AaLS-13 was assumed to consist of 180 monomers.[7]

Stopped-flow kinetics. A mixture of AaLS-13 and GFP(+36)-APEX2 was diluted to 10 nM on the basis of the heme absorption of GFP(+36)-APEX2 (ε_{405} = 128 000 $M^{-1}cm^{-1}$)[3] in PBS buffer (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 137 mM, KCl 2.7 mM pH 7.4). Substrate solutions containing 5, 10, 15, 20, 30, 40, and 60 mM guaiacol and 5 mM H_2O_2 were prepared in PBS buffer and used immediately. Solutions were equilibrated at 25 °C in the stopped-flow UV-vis spectrometer. Product formation was monitored by the change in absorbance at 470 nm.[3]

Typically, 3 to 5 shots were averaged and the averaged traces were fitted by linear regression using Kaleidagraph. The observed initial rates were fitted to the Michaelis-Menten equation.

Polymerization in solution. GFP(+36)-APEX2 was encapsulated in AaLS-13 (at an AaLS-13 monomer to GFP(+36)-APEX2 ratio of 10:1) in PBS buffer as described above, and the mixture was kept at rt for ca. 30 min. 100 μM H_2O_2 and 100 μM DAB were added to the encapsulation mixture (500 μL), and the reaction was monitored by UV-vis spectrophotometry. As a control, the same amount of substrate was also added to a solution of GFP(+36)-APEX2 enzyme only. After a reaction time of 1h at room temperature, the reaction was quenched by removing excess substrate by spinning the mixture through a desalting column (PD-Miditrap G25, GE Healthcare) using PBS as buffer. The capsid-polymer complexes were isolated by SEC using a Superose 6 column (running buffer: PBS) and analyzed by SDS-PAGE and UV-vis spectroscopy.

Dynamic light scattering (DLS) measurements. PolyDAB was produced in situ as described in the previous section using both encapsulated and free GFP(+36)-APEX2. The quenched polymerization reactions were analyzed without further purification by SEC. 50 μL of the reaction mixture was added to a plastic cuvette containg 50 μL PBS and DLS was performed at a fixed angle of θ = 173 °C. Each sample was measured three times. Analysis of the data was carried out using the Malvern Zetasizer software (Malvern, Worcestershire, UK). The average hydrodynamic diameter, polydispersity index (PDI) and particle distributions were determined by analysis of both volume (Figure 2b) and intensity (Figure S5). The two methods afforded parameters that were the same within experimental error.

Screening for polymerization conditions. GFP(+36)-APEX2 was encapsulated in AaLS-13 (at an AaLS-13 monomer to GFP(+36)-APEX2 ratio of 10:1, 25:1, 50:1 and 100:1) in PBS as described above, and the mixture was kept at rt for ca. 30 min. Screening was carried out as follows; A 96-well plate filled with 100 μL PBS containing 50, 100, 200, 300, 500, and 1000 μM DAB and 1000 μM H_2O_2. To each well was added 100 μL of AaLS-13/GFP(+36)-APEX2 complexes in PBS to give a final concentration of 1 μM in AaLS-13 monomer. The formation of precipitate was detected visually.

Monitoring of the polymerization reaction by transmission electron microscopy (TEM). Mixtures of AaLS capsid (50 μM with respect to monomer) and GFP(+36)-APEX2 at a AaLS monomer:enzyme ratio of 5:1 and 25:1 in 2xPBS (twice concentrated PBS) were prepared and incubated for ca. 30 min followed by dilution to a final AaLS monomer concentration of 3-5 μM which is ideal for observation by TEM. Additionally, a mixture of AaLS-13 capsids with APEX2 lacking the GFP(+36)-tag at a ratio of 5:1 and a solution of GFP(+36)-APEX2 of 1 μM in 2xPBS were prepared as controls. A glow-discharged EM grid was soaked with the encapsulation mixtures for 1 min to fix the enzyme-capsid complexes, washed two times with 2xPBS buffer, soaked in a substrate solution containing 1.5 mM DAB and 2.5 mM H_2O_2 in 2xPBS, and incubated at room temperature for the indicated times. The DAB/H_2O_2 mixture was always prepared fresh just before application to the grid. After incubating the grids with substrate, the polymerization reaction was stopped by washing the grids 5 times in buffer followed by staining with either uranyl acetate (2% in H_2O) for 30 sec or with OsO_4 (2% in H_2O) for 30-60 min. TEM pictures were recorded using a FEI Morgagni 268 transmission electron microscope. Particle size was determined by measuring the average diameter of 10 particles per image using ImageJ software.

Supporting Table

Table 1. List of primers used in this study.

Name	Sequence 5'-3'
MBP-for	CATATACTCGAGCATGAAAATCGAAGAAGGTAAACTGG
MBP-rev	CATAATACTAGTTAAGTCTGCGCGTCTTTCAGG
pAKZ3-for	GATCCCGCGAAATTAATACGACTCACTATAGG
pACYC-KpnI-rev	CTGCCACCGGGTACCTTGTAGCGTTCGTCGCGTCCGTG
pACYC-KpnI-for	GCTACAAGGTACCCGGTGGCAGCGGGGGCAGCGGC
APEX2-XhoI-for	GGCTCGAGCGGAAAGTCTTACCCAACTGTGAGTGC
APEX2-SpeI-rev	GCTGACTAGTTAGGCATCAGCAAACCCAAGCTCGG

Supporting data

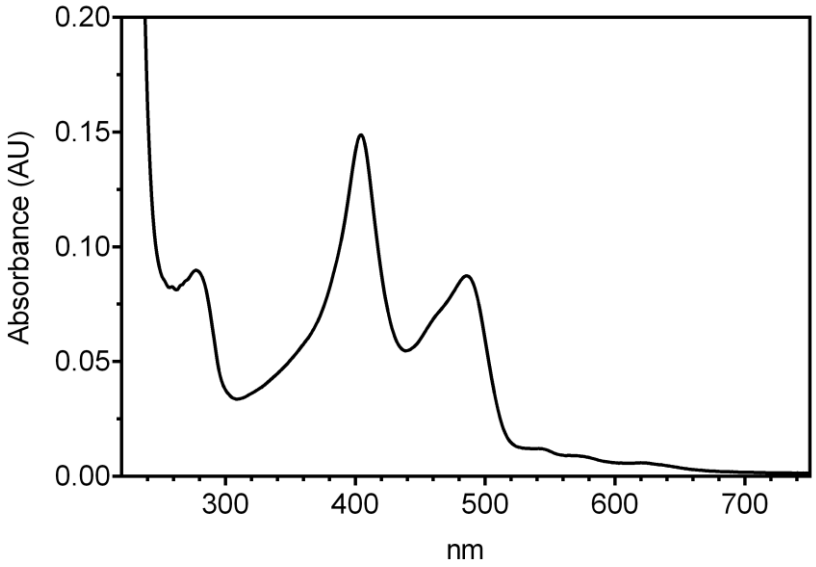

Figure S1. UV-vis spectrum of heme-reconstituted GFP(+36)-APEX2. Refer to the Experimental section on page S4 for the determination of heme occupancy.

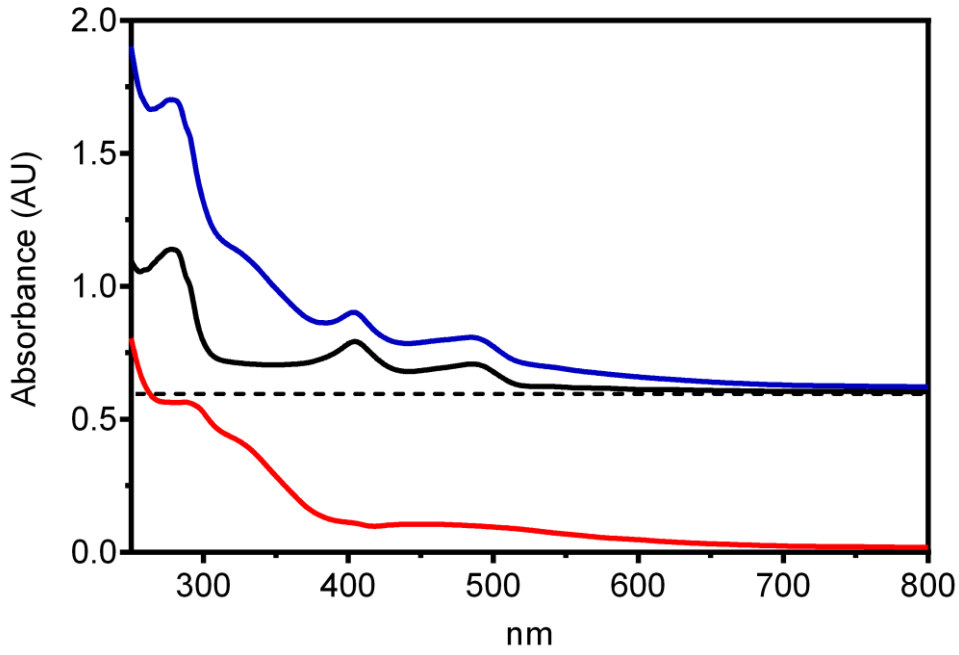

Figure S2. UV-vis absorption spectrum of a mixture of AaLS-13 and GFP(+36)-APEX2 at a 10:1 (capsid monomer: enzyme) ratio before (black) and 60 min after (blue) addition of the substrates DAB and H_2O_2 (100 μM each). The difference of the two spectra (red) shows a broad absorption between 300 and 700 nm with a maximum absorbance at ~300 nm, characteristic of polymerized DAB.[8]

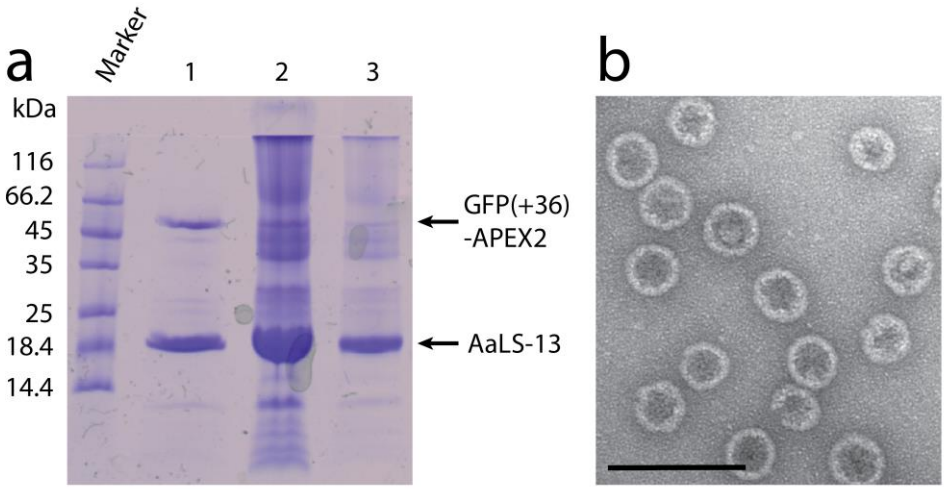

Figure S3. Analysis of the polymerization products. (a) SDS-PAGE (20%) of capsid-enzyme-polymer complexes. Lane 1: AaLS-13+GFP(+36)-APEX2; Lane 2: Capsid-APEX2 complexes that were reacted with 100 μM DAB + 100 μM H_2O_2 and purified via SEC. Lane 3: Same sample as in lane 2 but diluted 10-fold. Polymer products attached to GFP(+36)-APEX2 shift the apparent mass to higher molecular masses and prevent the fusion enzyme from entering the gel, explaining the absence of a clear band for GFP(+36)-APEX2 in lanes 2 and 3. (b) Negative stain electron microscopy images of the capsid-polymer particles produced under the same conditions as in (a). Scale bar: 100 nm.

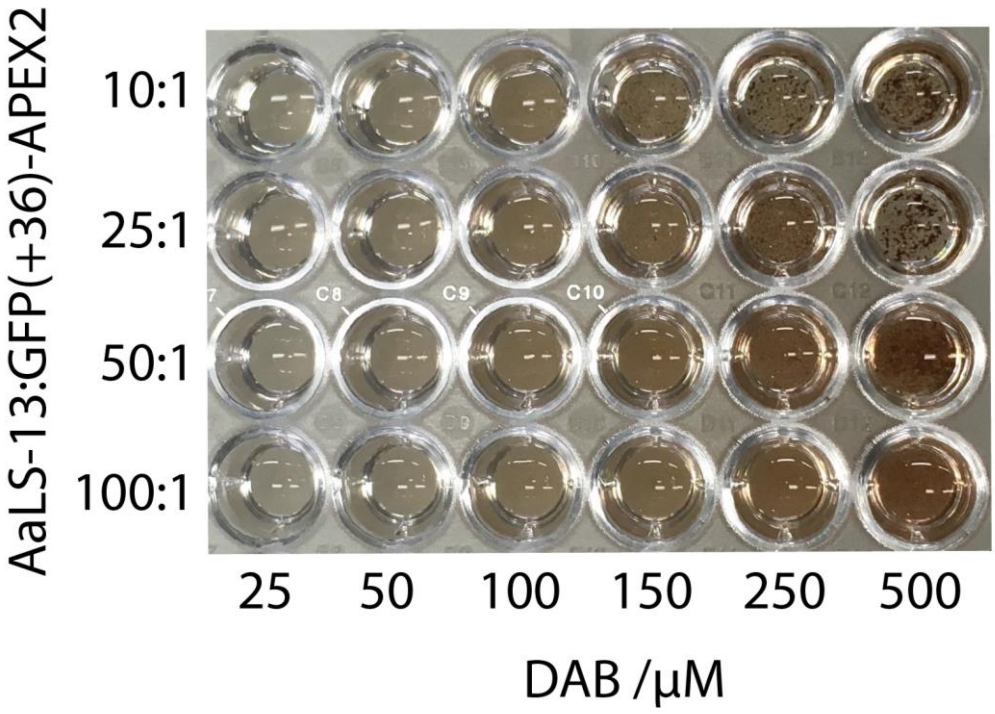

Figure S4. Screening conditions for polymerization as described in the materials and methods section; the concentration of H_2O_2 was kept constant at 500 µM. All wells contain the same amount of AaLS-13 (monomer concentration 1 µM) and the corresponding amount of GFP(+36)-APEX2 to give the AaLS-13 monomer:enzyme ratios indicated on the vertical axis. The plate was incubated at rt for 1 h. DAB concentrations >100 µM lead to the formation of precipitate.

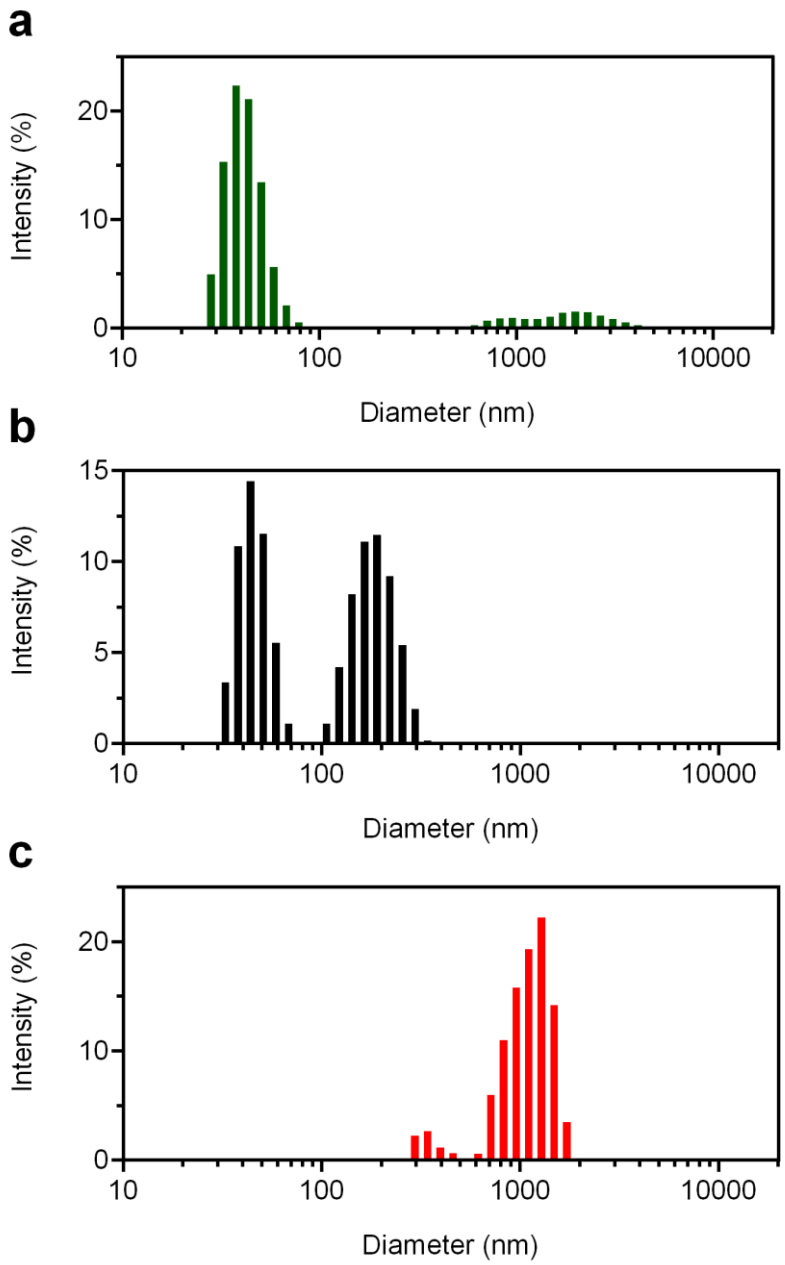

Figure S5. DLS intensity distribution analyses. (a) Empty AaLS-13 (Ø = 40 ± 6 nm). (b) AaLS-13-APEX2 complexes reacted with 100 µM DAB and H_2O_2 (Ø = 43 ± 7 nm). (c) PolyDAB products obtained without a capsid template (Ø = 874 ± 125 nm). The high molecular weight peak observed in b is more pronounced in the intensity distribution plot, compared to that in the volume distribution plot shown in Figure 2b because the particle scattering intensity is proportional to the square of the molecular weight. The volume distribution plots thus provide a more accurate representation of the relative populations of particles in our samples.

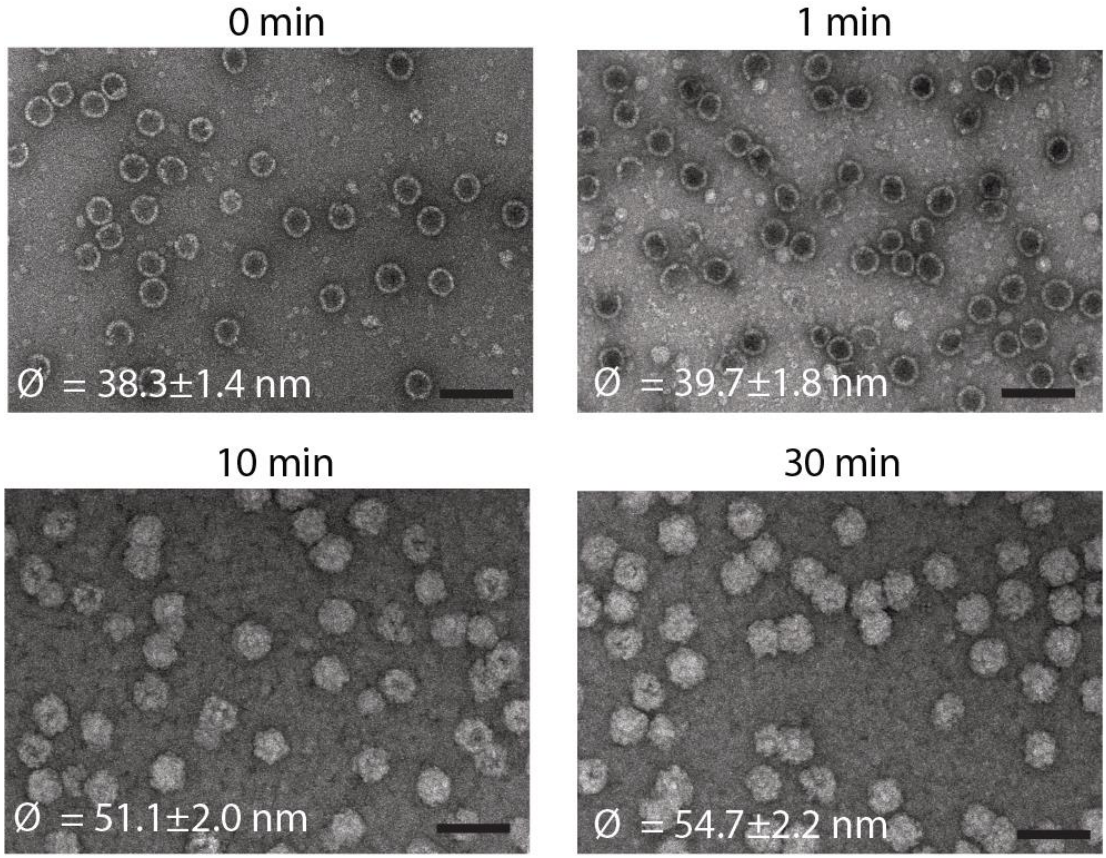

Figure S6. EM images of negatively stained polymeric nanoparticles at low APEX2 loading. AaLS-13 capsid and GFP(+36)-APEX2 were mixed in a 25:1 ratio which corresponds to approximately 3 heme-containing APEX enzymes per 180-mer capsid. The enzyme-capsid complexes were fixed on the EM grid and treated with DAB and H_2O_2 (1.5 and 2.5 mM, respectively) for various times. Scale bars: 100 nm.

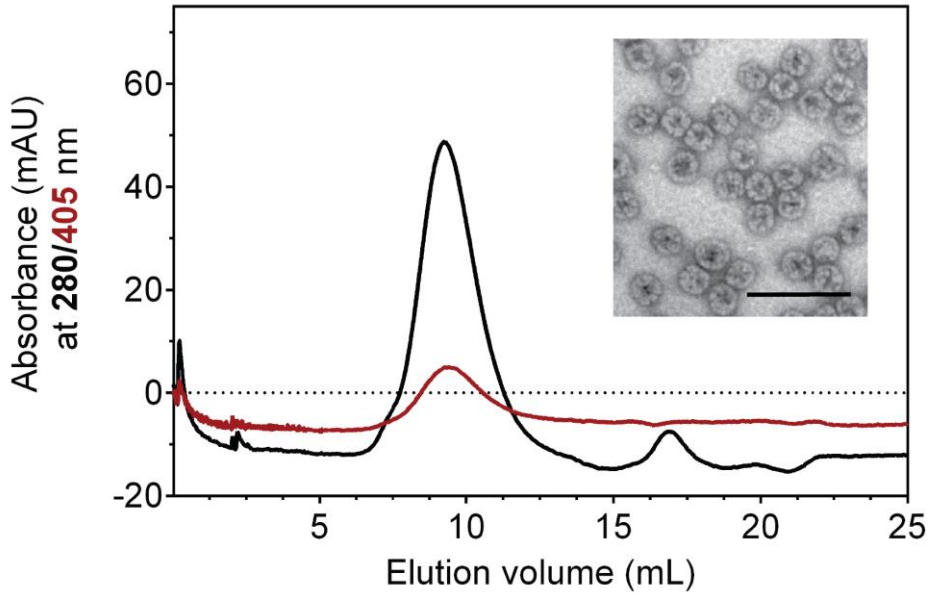

Figure S7. SEC trace of AaLS-neg/GFP(+36)-APEX2 complex. Purified AaLS-neg capsids (50 μM) were mixed with GFP(+36)-APEX2 (5 μM) in 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and 1 mM EDTA followed by purification by SEC on a Superose 6 column. Inset: negative stain EM image of the eluted capsid fraction. Scale bar: 100 nm. The purified particles were subsequently used for templated polymerization of DAB.

References

1 Y. Azuma, R. Zschoche, M. Tinzl and D. Hilvert, *Angew. Chem. Int. Ed.*, 2016, **55**, 1531–1534.

2 R. K. Jha, A. Leaver-Fay, S. Yin, Y. Wu, G. L. Butterfoss, T. Szyperski, N. V. Dokholyan and B. Kuhlman, *J. Mol. Biol.*, 2010, **400**, 257–270.

3 S. S. Lam, J. D. Martell, K. J. Kamer, T. J. Deerinck, M. H. Ellisman, V. K. Mootha and A. Y. Ting, *Nat. Methods*, 2015, **12**, 51–60.

4 I. Barr and F. Guo, *Bio-protocol*, 2015, **5(18)**, e1594.

5 F. P. Seebeck, K. J. Woycechowsky, W. Zhuang, J. P. Rabe and D. Hilvert, *J. Am. Chem. Soc.*, 2006, **128**, 4516–7.

6 T. Beck, S. Tetter, M. Künzle and D. Hilvert, *Angew. Chem. Int. Ed.*, 2015, **54**, 937–940.

7 B. Wörsdörfer, K. J. Woycechowsky and D. Hilvert, *Science*, 2011, **331**, 589–92.

8 J. Cohen, *Anal. Biochem.*, 1973, **53**, 208–222.