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Cytochrome *c*-poly(acrylic acid) conjugates with improved peroxidase turnover number

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Supporting Information

Materials and Methods

Materials

Cytochrome *c* and horseradish peroxidase (230 U mg^{-1}) were purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). Poly(acrylic acid) 1.8k, 8k, 100k, and 250k g mol^{-1} (weight average molecular weight), sodium phosphate, Brilliant blue R250, bromophenol blue, guaiacol, agarose Type I (low EEO), glycine, chloroform, acetic acid, isopropanol, acrylamide, sodium dodecyl sulfate, and trinitrobenzene sulfonic acid (5% w/v in water) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(acrylic acid) 50k (weight average molecular weight) was purchased from Polysciences, Inc (Warrington, PA). Tris base, N,N'-methylenebis(acrylamide), ammonium persulfate, tetramethylenediamine, and 2-mercaptoethanol were purchased from Thermo Fisher Scientific (Waltham, MA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from TCI America (Portland, OR). Poly(ethylene glycol), 20000 g mol^{-1}, was purchased from Alfa Aesar (Haverhill, MA). Hemoglobin was purchased from MP Biomedicals (Santa Ana, CA). Dialysis membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

Synthesis of enzyme-PAA conjugates

Protein-enzyme conjugates were prepared per previously reported methods,[1] with a few minor modifications. First, activated poly(acrylic acid) solution was prepared by stirring PAA with EDC in sodium phosphate buffer (20 mM pH 7.0) for 10 minutes. Next, cyt *c*, HRP, or Hb were added while stirring, and the resulting mixture was reacted at room temperature for four hours. The final concentration of enzyme and PAA were 10 µM. The concentration of EDC was maintained at 5 mM for all M_w of PAA to avoid excessive enzyme-enzyme crosslinking, with the exception of 250k PAA. Here, 10 mM EDC was required to obtain complete crosslinking and ensure there was no free cyt *c*, as determined by gel electrophoresis. The EDC reaction byproducts were removed by dialysis against 20 mM sodium phosphate, pH 7.0, using a 200-500 Da cutoff membrane for cyt *c*-PAA(1.8k) and cyt *c*-PAA(8k), and a 15 kDa cutoff membrane for all other M_w PAA.

Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose (0.5% w/v) in Tris-acetate buffer (40 mM, pH 7.0). Solutions were heated in the microwave for 30 s to dissolve agarose. Samples were prepared for electrophoresis by combining 20 µL of 10 µM cyt *c* or cyt *c*-PAA solution with 10 µL of loading buffer (50% v/v glycerol, 0.01% w/v bromophenol blue). Gels were run in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc., Grand Island, NY) for 30 minutes, with 100 V constant voltage and using 40 mM Tris acetate, pH 7.0 as the running buffer. Gels were stained for at least four hours with an aqueous solution of 20% v/v acetic acid and 0.03 g L^{-1} Brilliant blue R250, then destained for four hours with 10% v/v acetic acid prior to imaging.

SDS-PAGE

12.5% w/w acrylamide separating gels and 5% w/w acrylamide stacking gels were used for SDS-PAGE. The separating gel was prepared by combining 4.2 mL 30% acrylamide solution (18.75 g acrylamide, 0.5 g N,N'-methylenebis(acrylamide), 64 mL DI water), 2.5 mL 4x lower gel buffer (9.35g Tris base, 0.2 g SDS, 50 mL DI water, pH 8.8), 3.3 mL DI water, 50 µL 10% ammonium persulfate, and 5 µL tetramethylenediamine. The stacking gel was prepared by combing 0.85 mL 30% acrylamide solution, 1.25 mL 4x upper gel buffer (3.025 g Tris base, 0.2 g SDS, 50 mL DI water, pH 6.8), 2.9 mL DI water, 25 µL 10% ammonium persulfate, and 5 µL tetramethylenediamine. The separating gel was poured into a 1 mm mold and allowed to polymerize for 30 minutes, after which the stacking gel was poured and similarly allowed to polymerize.

Samples were prepared by combining 20 µL of ~50 µM cyt *c* with 15 µL SDS-PAGE loading buffer (2% w/v SDS, 10% w/w 2-mercaptoethanol), after which the solutions were heated at 90 °C for 2 minutes. 15-20 µL of sample solution was loaded per well. Gels were run in a vertical Bio-Rad Mini-PROTEAN electrophoresis apparatus at 150 V for 15 minutes, then 200 V until the dye front reached the bottom of the gel (~20 minutes). The running buffer was 3.03 g L^{-1} Tris base, 14.41 g L^{-1} glycine, and 1 g L^{-1} SDS. The gel was stained with an aqueous solution of 10% v/v acetic acid, 10% v/v isoproponal, and 0.02 g L^{-1} Brilliant blue R250 for four hours, then with an aqueous solution of 20% v/v acetic acid and 0.03 g L^{-1} Brilliant blue R250 for four hours. Gels were destained with 10% acetic acid prior to imaging.

Trinitrobenzene sulfonic acid assay

Quantitation of primary amines using trinitrobenzene sulfonic acid (TNBSA) was performed by a previously reported method.[2] 250 µL of 0.01% (w/v) TNBSA was added to 500 µL of protein or glycine solution and incubated for 2 hours at 37 °C. After incubation, 250 µL 10% (w/v) sodium dodecyl sulfate and 125 µL of 1 M HCl were added to each sample. The absorbance at 335 nm was then obtained. To produce the standard curve, glycine solutions with concentrations of 1, 3, 5, 7, 10, 15, and 20 µM were

analyzed in this way. Protein samples were prepared at 0.8 µM. The concentration of primary amines in each protein sample was determined using the glycine calibration plot (one 1° amine per glycine), and the number of primary amines per protein was determined by dividing the concentration of primary amines by the protein concentration.

Dynamic light scattering

Conjugate hydrodynamic radius was measured by dynamic light scattering (DLS). Samples were filtered with a 0.22 µm syringe filter to remove dust particles and large aggregates prior to analysis. Precision Detectors PDDLS/CoolBatch 40T and PD4047 dynamic light scattering detectors with 658 nm laser and 90° laser and monitoring optics were employed for DLS measurements. Measurements were completed in triplicate.

Circular dichroism spectroscopy

Circular dichroism spectra were measured with a JASCO J-710 spectropolarimeter. UV CD spectra were scanned from 195-260 nm using a 0.05 cm path length quartz cuvette and the following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 2 nm bandwidth, 6 scans per spectrum. Soret CD spectra were scanned from 300-550 nm using a 1 cm path length quartz cuvette and the following parameters: 1 nm data pitch, continuous scanning mode, 1 second response speed, 1 nm bandwidth, 10 scans per spectrum. Because the Soret CD features are weak, mild smoothing was applied (minimum data convolution width) to improve noise using JASCO Spectra Analysis software. Blank spectra (buffer solution only) were subtracted from all spectra, which were then normalized with respect to path length and protein concentration.

UV/Visible absorbance spectroscopy

Soret and Q band absorbance spectra were obtained using a HP 8453 UV/visible spectrophotometer and a 1 cm pathlength quartz cuvette. The change in Q band absorbance in the presence of H_2O_2 was monitored by injecting 100 µM H_2O_2 into a solution of 10 µM cyt *c* or cyt *c*-PAA, then recording the UV/Vis spectrum at intervals. Delta absorbance plots were produced by subtracting the spectrum of a given time point from the spectrum of the subsequent time point. Measurements were completed in triplicate.

Michealis-Menten kinetics and activity studies

Michealis-Menten kinetics. Solutions containing cyt *c* or cyt *c*-PAA, guaiacol, and sodium phosphate buffer (pH 7.4) were prepared and placed in a 1 cm glass cuvette. Absorbance at 470 nm (A_{470}) was monitored as a function of time using a HP 8453 UV/visible spectrophotometer. Once a stable baseline was established, H_2O_2 was injected into the cuvette while stirring at 1000 RPM, and the change in A_{470} was recorded for 1 minute. The final concentrations of reactants were: [cyt *c*] = 1 µM, [guaiacol] = 5-200 µM, [H_2O_2] = 50 mM, and [sodium phosphate] = 20 mM. The activity assay was performed in triplicate at 20 °C and atmospheric pressure for each concentration of guaiacol tested. Initial rates were obtained from the slope of the linear portion of the kinetic traces, where the change in absorbance with respect to time was the greatest. The variation of initial rate with guaiacol concentration was fit to the Michealis-Menten model and the Michealis constants were extracted, as described below.

The Michaelis-Menten model indicates that enzymatic catalysis occurs in two steps, a reversible binding of substrate to enzyme followed by conversion of substrate to product and subsequent release of product. The Michaelis-Menten equation

$$v_0 = \frac{v_{max}[S]}{K_M + [S]}$$

is a hyperbolic function that describes how the initial rate of the reaction depends on the concentration of substrate [S], the maximum initial rate v_{max}, and the affinity of the enzyme for the substrate K_m (the Michaelis constant). A decrease in K_M indicates stronger substrate binding and thus higher affinity.[3] The turnover number, k_{cat}, was equal to $v_{max}/[cyt\ c]$, and was the rate constant for the conversion of enzyme-substrate complex to product.[3] The catalytic efficiency was equal to k_{cat}/K_M. The peroxidase activity assay employed here required two substrates, guaiacol and H_2O_2. In this case, H_2O_2 was kept in excess, and thus the observed K_M, v_{max}, and k_{cat} are apparent parameters with respect to guaiacol.

Michealis-Menten kinetics of HRP-PAA and Hb-PAA were obtained using the same method. Final activity assay concentrations were: [HRP] = 20 nM, [guaiacol] = 1-16 mM, [H_2O_2] = 0.2 mM, [sodium phosphate] = 20 mM; or [Hb] = 1 µM, [guaiacol] = 0.1-16 mM, [H_2O_2] = 50 mM, [sodium phosphate] = 20 mM.

Activity studies at various pH. Full kinetic assays were not completed to study the effect of pH or local crowding agents. Instead, conditions were selected where [guaiacol]>>K_M so that the initial rate could be seen as an approximation of v_{max}. As such, activity studies were performed follow the same method as the kinetic studies, with the following concentrations: [cyt *c*] = 1 µM, [guaiacol] = 200 µM, [H_2O_2] = 50 mM, and [sodium phosphate] = 20 mM. For pH dependent studies, the pH of sodium phosphate buffer was varied from 6-8. In all cases, activity studies were completed in triplicate.

Guaiacol partitioning studies

Partitioning of guaiacol to the polymer phase was estimated by examining the partitioning of guaiacol between chloroform and buffered aqueous solutions of poly(acrylic acid). 2 mM guaiacol was dispersed in 20 mM sodium phosphate, pH 7.0 in a glass vial, an equal volume of chloroform was added. For testing of partitioning to PAA phase, 10 µM PAA was also added to the phosphate buffer and guaiacol solution. The vial was capped and shaken vigorously, after which the chloroform and aqueous layers were

allowed to separate. This was repeated three times, then the upper aqueous layer was removed. The concentration of guaiacol in the aqueous layer was determined from the UV/Vis spectrum using the extinction coefficient at 274 nm, $\varepsilon = 2550\ M^{-1}\ cm^{-1}$.[4] Samples were prepared and analyzed in this manner in triplicate.

Estimation of Debye length

When ions are dispersed in solution, their charges can be screened by other charged species in the solution. The strength of electrostatic interactions with a charge decay approximately exponentially with distance, and the Debye length (κ^{-1}) is the characteristic length of this decay.[5,6] The Debye length can be calculated theoretically using the equation:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_r \varepsilon_0 k_b T}{\sum n_i z_i^2 e^2}}$$

where ε_r is the dielectric constant of the solvent, ε_0 is the permittivity of free space, k_b is the Boltzmann constant, T is the temperature, e is the electronic charge, and n_i and z_i are the number density concentration and charge of the *i*th species in solution.[6] This equation was employed to estimate the Debye length under the conditions of the kinetic assay (20 mM sodium phosphate, pH 7.0, 20 °C). Contributions from carboxylate groups of PAA were neglected, as the concentration of these groups was one or more orders of magnitude smaller than that of the buffer ions in all cases (1 µM cyt c-PAA).

Heme bleaching studies

Bleaching of the cyt *c* heme was examined by addition of hydrogen peroxide to cyt *c* in the absence of guaiacol. 1 µM cyt *c* or cyt *c*-PAA was prepared in 20 mM sodium phosphate, pH 7.0. Solutions were stirred at 20 °C, and 50 mM H_2O_2 was added. The absorbance at 410 nm was monitored as a function of time after addition of peroxide.

References

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2. A. F. Habeeb, *Anal. Biochem.*, 1966, **14**, 328–336.
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Supplementary data figures

Agarose gels of physical mixtures

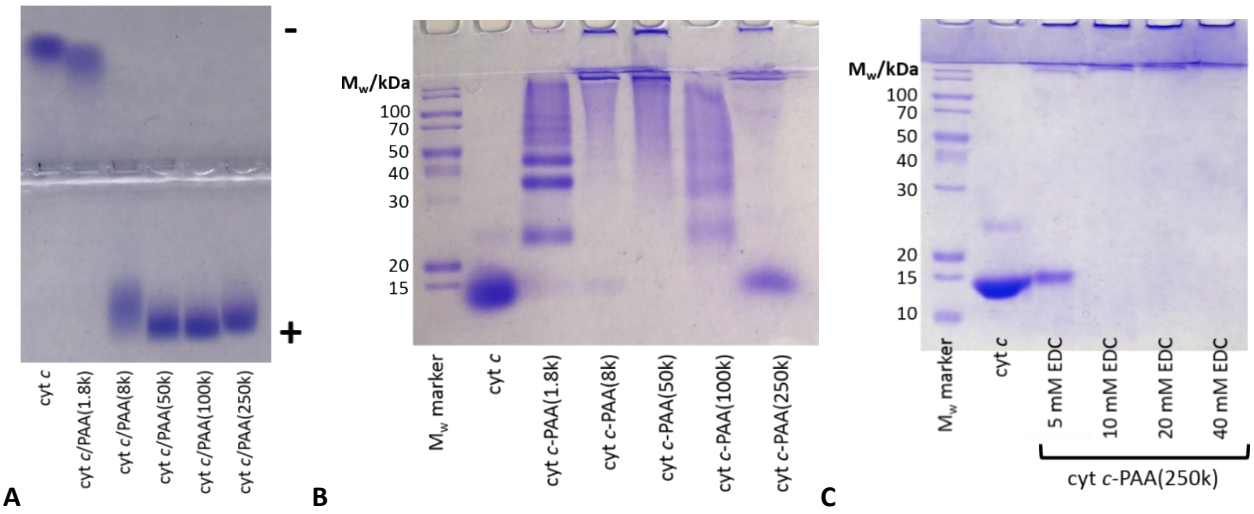


Figure S1. (A) Agarose gel of cyt *c*/PAA physical mixtures revealed strong complexation between cationic cyt *c* and anionic PAA for PAA(8k) and larger M_w. **(B)** SDS-PAGE gel of cyt *c*-PAA conjugates synthesized with 5 mM EDC. Significant unconjugated cyt *c* was found in cyt *c*-PAA(250k) with 5 mM EDC. **(C)** SDS-PAGE gel of cyt *c*-PAA(250k) with increasing [EDC]. 10 mM EDC was sufficient to obtain complete crosslinking.

Quantification of primary amines

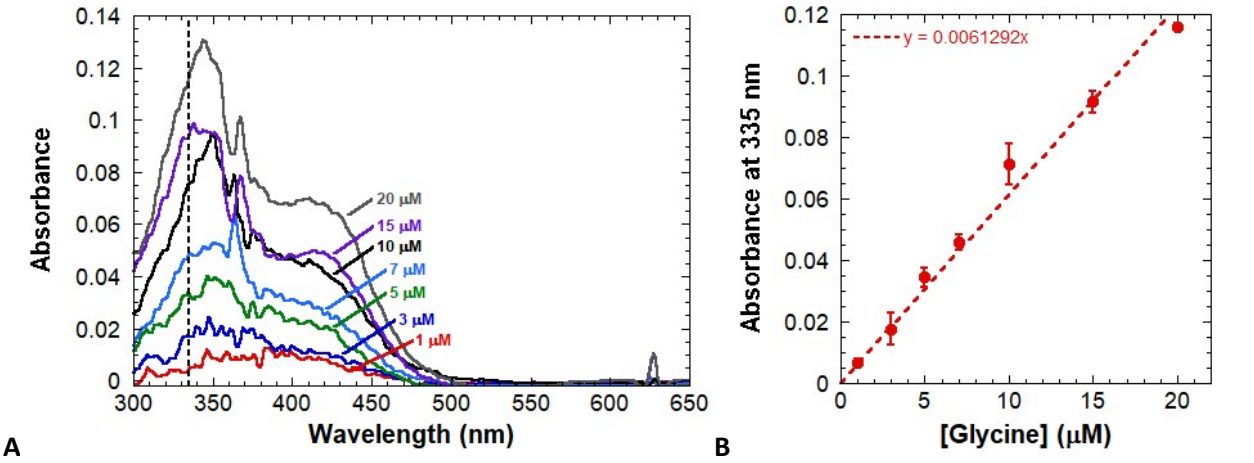


Figure S2. (A) Absorbance spectra of glycine standards incubated (37 °C, 2h) with trinitrobenzene sulfonic acid (TNBSA). **(B)** Calibration curve for concentration of primary amines produced from spectra in panel A.

Sample	1° amines/cyt *c*
cyt *c*	22.9(±0.8)
cyt *c*-PAA(1.8k)	9.7(±0.6)
cyt *c*-PAA(8k)	10.2(±0.4)
cyt *c*-PAA(50k)	12(±1)
cyt *c*-PAA(100k)	11(±2)
cyt *c*-PAA(250k)	13.1(±0.6)

Table S1. Number of primary amines per cyt *c* as determined from the TNBSA assay.

Physical mixture MM kinetics plots

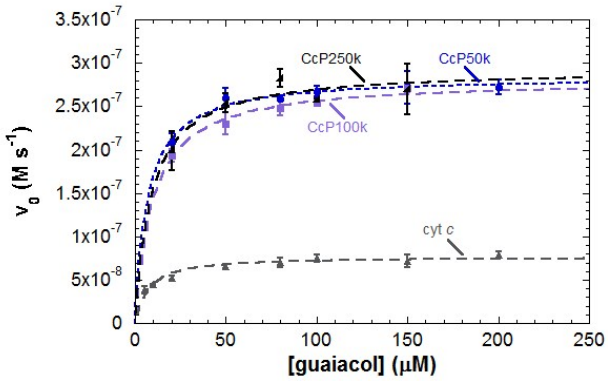


Figure S3. Michealis-Menten kinetics of physical mixtures of cyt *c* and PAA 50k, 100k, or 250k. The behavior of cyt *c*/PAA(50k) was similar to its corresponding covalent conjugate. No additional kinetic enhancement was noted in the physical mixtures with 100k or 250k relative to cyt *c*/PAA(50k), unlike the covalent conjugates with the same M_w PAA.

Tandem and physical mixture CD

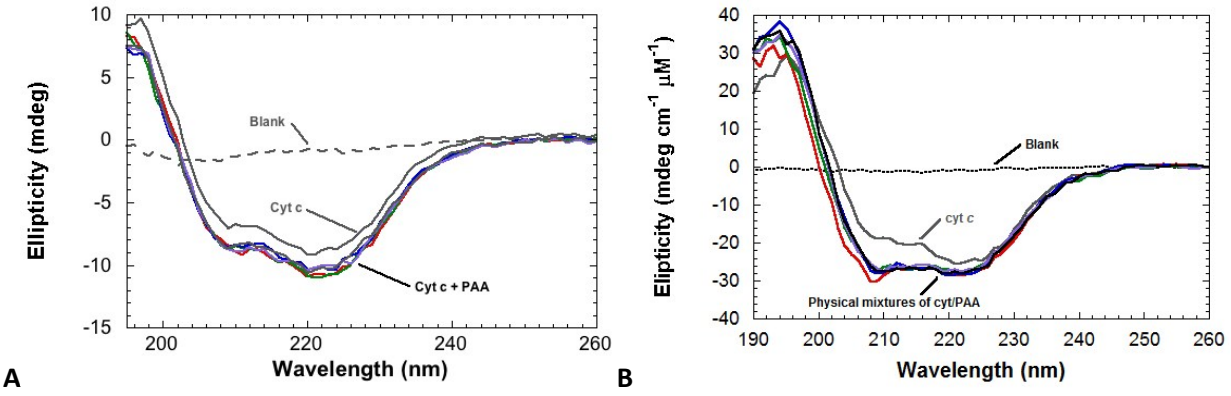


Figure S4. (A) Circular dichroism spectra of cyt *c* and PAA in tandem cells. Cyt *c* and PAA were present in separate cuvettes, which were placed together in the spectrometer. This revealed that scattering or

absorbance from PAA in the UV region caused an increase in the observed molar ellipticity of cyt *c*, even when PAA was present in a separate cell and thus had no direct interaction with cyt *c*. **(B)** UV CD spectra of physical mixtures of cyt *c* and PAA (no EDC).

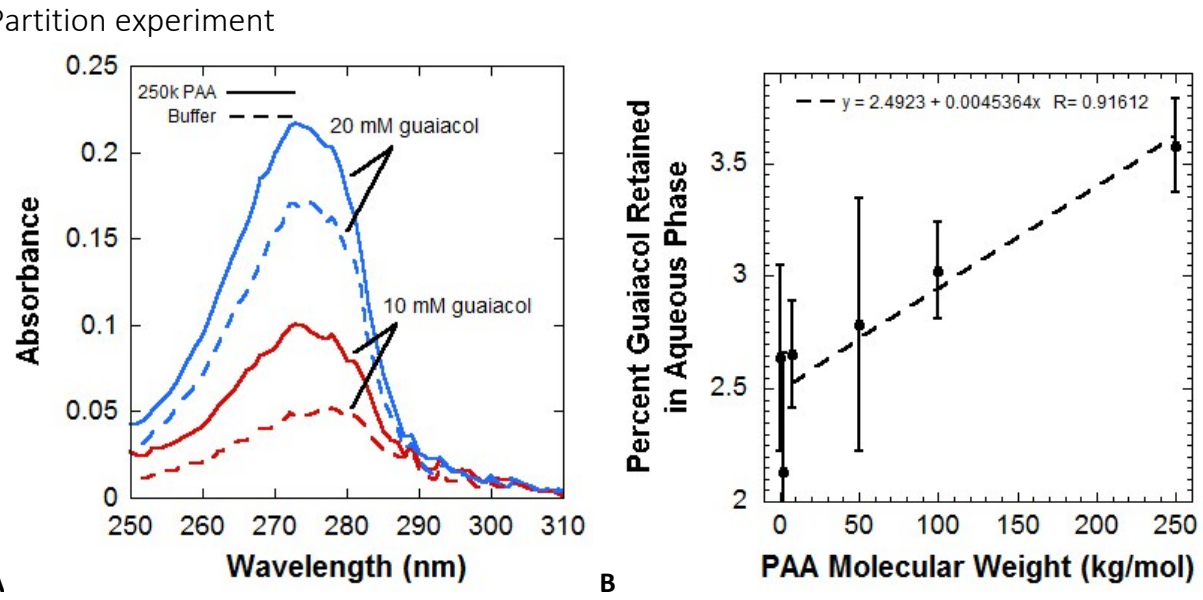


Figure S5. (A) Absorbance spectra of guaiacol retained in the aqueous phase after extraction with chloroform (initial [guaiacol] in aqueous phase was 2 mM). The solid line represents the aqueous phase containing 250k PAA in sodium phosphate buffer, pH 7.0, while the dashed line represents the aqueous phase which contained only the buffer. **(B)** Percent retention of guaiacol in aqueous phase as a function of PAA molecular weight. Percent retention was calculated from the absorbance spectra after extraction with chloroform.

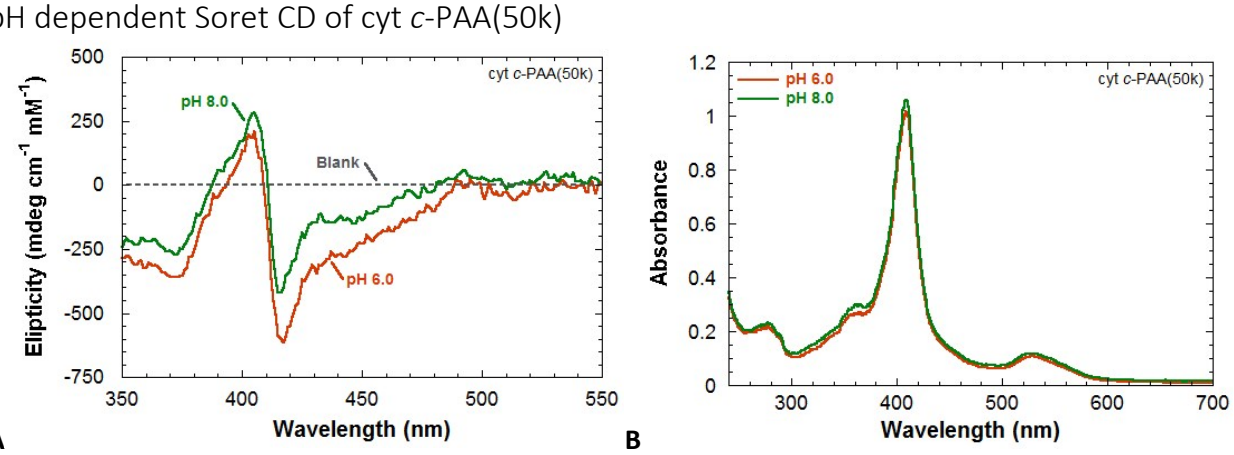
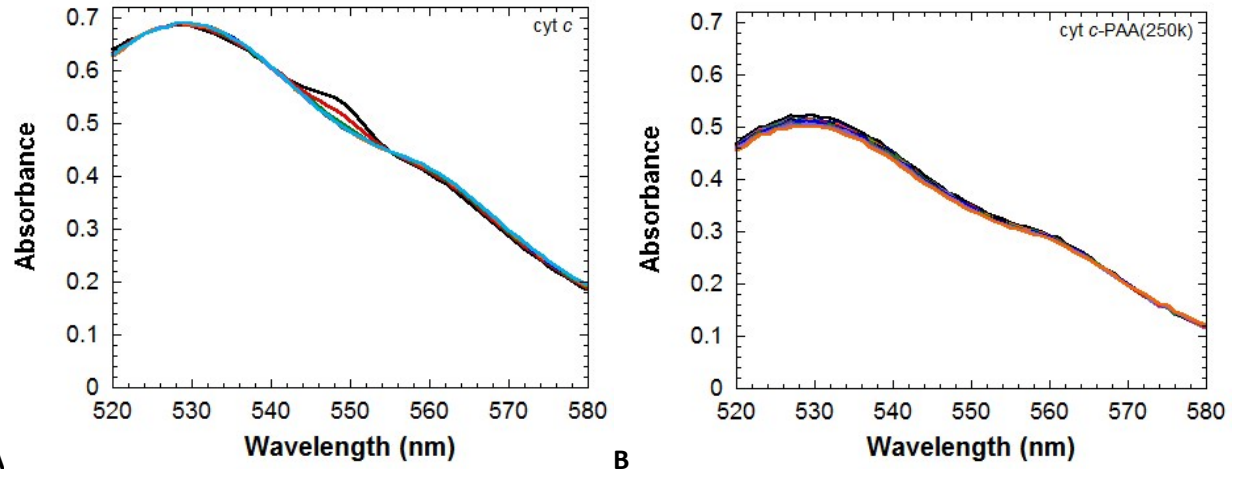


Figure S6. (A) Soret CD of cyt *c*-PAA(50k) at pH 6.0 (orange curve) and pH 8.0 (green curve). Changes in the relative intensities of the peaks indicates small changes in heme solvent exposure, while the presence of both positive and negative peaks indicated heme ligation did not change when the pH was changed.

(B) Heme absorbance of cyt *c*-PAA(50k) at pH 6.0 (orange curve) and pH 8.0 (green curve). Soret and Q band maxima were found in the same position at each pH, indicating heme ligation was unchanged and heme iron was in the native, ferric state.

Spectra used to produce delta absorbance plots



A **B**

Figure S7. (A) Spectra of the Q band of cyt *c* in the presence of 100 µM H_2O_2. The shoulder at ~550 nm disappeared, while the absorbance at 533 and 553 nm increased, indicative of the formation of Compound III. **(B)** Spectra of Q band of cyt *c*-PAA(250k) in the presence of 100 µM H_2O_2. Unlike cyt *c*, no changes were noted after the addition of H_2O_2.

Heme bleaching studies

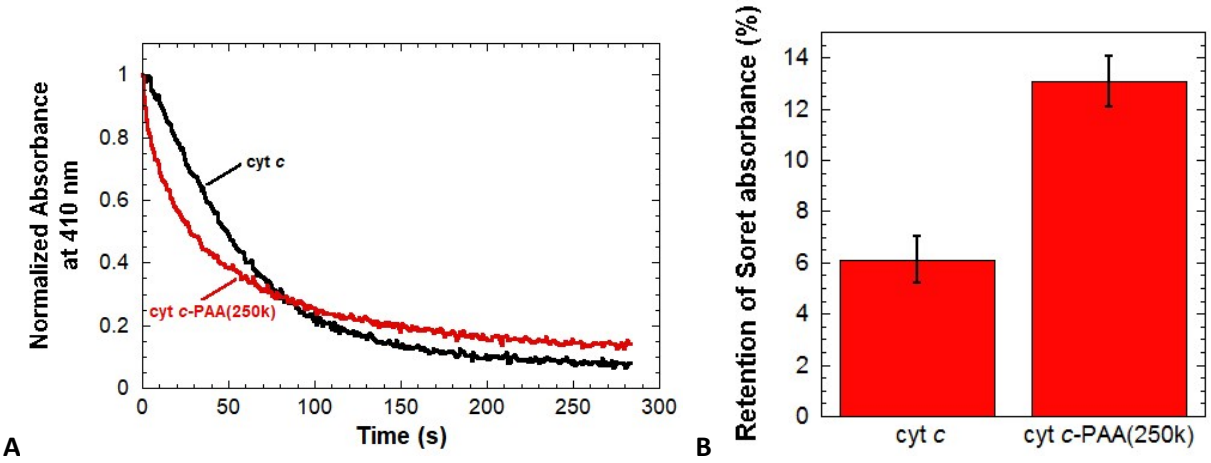


Figure S8. (A) Kinetic trace of cyt *c* heme bleaching (20 mM phosphate, pH 7.0, 20 °C). In the presence of 50 mM H_2O_2 but the absence of guaiacol, auto-oxidation and heme bleaching occurred, indicated by a decrease in the Soret absorbance at 410 nm. Decay of the heme in cyt *c*-PAA(250k) was initially more rapid than cyt *c*, consistent with the increased peroxidase activity caused by acidification of the local environment. However, bleaching of cyt c-PAA(250k) slowed significantly relative to cyt *c* as the reaction progressed. **(B)** After completion of the heme bleaching reaction, cyt *c*-PAA(250k) retained a greater degree of its Soret absorbance, indicating a lesser extent of inactivation in cyt *c*-PAA(250k).

Agarose gels of HRP-PAA and Hb-PAA

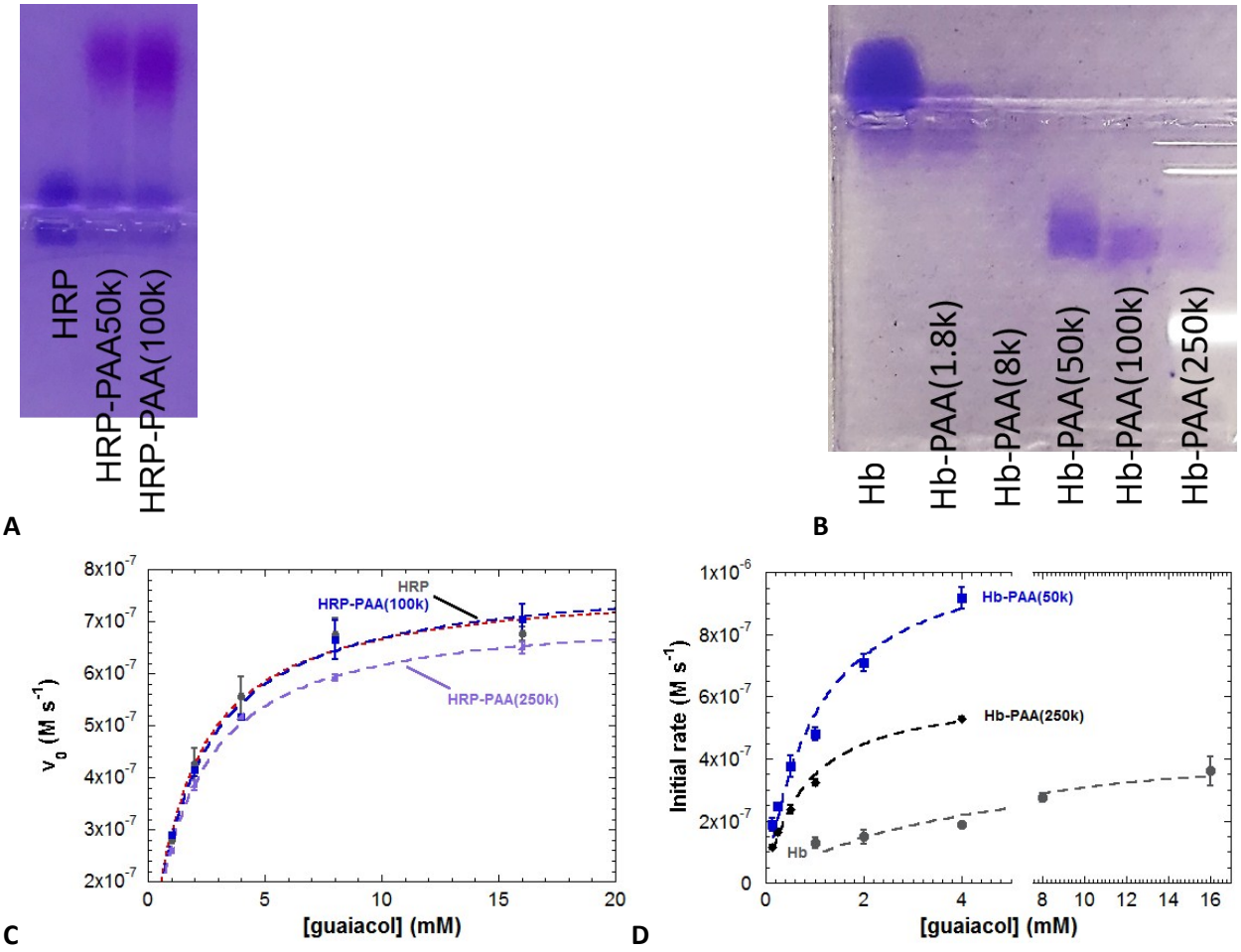


Figure S9. (A) Agarose gel (0.5% agarose w/w, pH 7.0) of HRP and HRP-PAA derivatives. **(B)** Agarose gel (0.5% agarose w/w, pH 7.0) of Hb and Hb-PAA derivatives. **(C)** Michealis-Menten kinetics of HRP-PAA (20 nM HRP, 200 μM H_2O_2) were nearly unchanged when conjugated to PAA. **(D)** Marked changes were noted in Hb-PAA Michaelis-Menten kinetics. K_M decreased sharply, while v_{max} increased with M_w PAA.

Sample	K_M (mM)	v_{max} (M s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
HRP	1.6(±0.2)	$7.8(\pm0.3)\times10^{-7}$	77(±3)	$4.7(\pm0.7)\times10^{4}$
HRP-PAA(100k)	1.8(±0.2)	$7.9(\pm0.2)\times10^{-7}$	79(±3)	$4.3(\pm0.5)\times10^{4}$
HRP-PAA(250k)	1.73(±0.09)	$7.2(\pm0.1)\times10^{-7}$	72(±1)	$4.2(\pm0.2)\times10^{4}$
Hb	4(±1)	$4.3(\pm0.6)\times10^{-7}$	0.43(±0.06)	$1.1(\pm0.4)\times10^{2}$
Hb-PAA(50k)	1.1(±0.3)	$11(\pm1)\times10^{-7}$	1.1(±0.1)	$11(\pm3)\times10^{2}$
Hb-PAA(100k)	0.8(±0.1)	$6.2(\pm0.4)\times10^{-7}$	0.62(±0.04)	$7.9(\pm0.1)\times10^{2}$

Table S2. Michealis-Menten parameters of HRP-PAA and Hb-PAA (shaded cells).