

Supplementary Material

Constructing Catalytic Antimicrobial Nanoparticles by Encapsulation of Hydrogen Peroxide Producing Enzyme Inside the P22 VLP.

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Materials and Methods

Materials. DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA) and Promega (Madison, Wisconsin). DNA primers were purchased from Eurofins MWG Operon (Huntsville, AL). *E. coli* BL21(λ DE3) and XL1 electrocompetent cells were purchased from Agilent Technologies (Santa Clara, CA). *P. furiosus* DNA was purchased from ATCC (Manassas, VA). QIAquick gel extraction kit and QIAprep Spin Miniprep kit were purchased from Qiagen (Valencia, CA). Lysozyme and NADH were purchased from Sigma Aldrich (St. Louis, MO). All other chemical reagents were purchased from Fisher Scientific (Pittsburgh, PA).

DNA molecular biology. Construction of free NADH oxidase was accomplished by digesting the pETDuet assembler vector with NcoI, BamHI, and SacHF then ligation (using T4 DNA Ligase from Promega) of NcoI and BamHI digested, phosphatased (NEB Antarctic Phosphatase) pRSF assembler vector previously described^[1]. Colonies resulting from transformation of the ligation were screened by colony PCR, restriction enzyme digestion and all hits were sequenced (Seqwright, Tx) for complete verification. After verifying of the correct sequence, DNA was transformed into BL21(DE3) for expression.

Protein expression and purification. *E. coli* strains harboring expression constructs were grown on LB medium at 37 °C in the presence of either kanamycin (free NADH oxidase) or ampicillin (P22-NADH oxidase) to maintain selection for the plasmid. Expression of the genes were induced by addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM once the cells reached mid-log phase ($OD_{600}=0.6$). Cultures were grown for 4.5 hours after addition of IPTG, and then the cells were harvested by centrifugation and cell pellets stored at -20 °C overnight.

Both encapsulated NADH oxidase (P22-NOX) and free NADH oxidase (NOX) cell pellets were resuspended in Lysis buffer (50 mM sodium phosphate, 25 mM sodium chloride, pH 7.5). 1 tablet per 10 mL of Pierce™ Protease Inhibitor Mini Tablets was dissolved in NOX cell pellet resuspension. 0.09 mg/mL Lysozyme, 0.13 mg/mL DNase, and 0.19 mg/mL RNase were added to samples and incubated at 37°C with shaking for 30 minutes. Cell suspensions were lysed by sonication. NOX cell suspension was incubated in a 70°C water bath for 20 minutes to denature and remove non-hyperthermophilic protein. Cell debris was removed by centrifugation at 12,000 g for 45 min at 4 °C. The supernatant was decanted and spun an additional 45 minutes at 12,000 g to remove any excess cellular debris. P22-NOX was further purified from the supernatant by ultracentrifugation over a 35% (w/v) sucrose cushion. The resulting viral pellet was resuspended in PBS (50 mM sodium phosphate, 25 mM sodium chloride, pH 7.5) and then purified over an S-500 Sephacryl size exclusion column using an ÄKTA Pharmacia FLPC. Flow rate for SEC purification was 1 mL/min of PBS. Fractions taken from SEC containing P22 were concentrated by ultracentrifugation and the resulting viral pellet was resuspended in an adequate volume of PBS. NOX was purified once each over 10 mL and 1 mL Ni-NTA columns at a 1 mL/min flow rate into 1 mL fractions. Protein was eluted with a linear 20-500 mM imidazole gradient over each column with an additional step gradient over the 1 mL Ni-NTA column at 50 mM imidazole to remove non-specific binders. Protein was dialyzed into 50 mM sodium phosphate, 25 mM sodium chloride, pH 7.5 and concentrated using a 10,000 Da molecular weight cut off filter. Concentrations for the P22 construct were determined by UV absorption measured at 280 nm assuming a molar extinction coefficient $\epsilon_{280} = 44,380 \text{ M}^{-1}\text{cm}^{-1}$ for coat protein and $\epsilon_{280} = 27,975 \text{ M}^{-1}\text{cm}^{-1}$ for NOX-SP (calculated using the ExPASy ProtParam tool, Swiss Institute of Bioinformatics) and the ratio of enzyme-SP fusion protein to Coat Protein was determined from the molecular weight determined from size exclusion chromatography as described previously^[2]. Because the final free NADH oxidase sample was not 100% pure, concentration was determined using densitometry and bicinchoninic acid (BCA) assays. Densities were measured with an AlphaImager 2200 using AlphaEaseFC software version 3.2.1. Three concentrations of NOX were run on SDS-PAGE and the densities corresponding to the 50 kDa molecular weight band were recorded and averaged. Total protein concentration was determined using the Pierce® BCA Protein Assay Kit from Thermo Scientific and multiplied by the densitometry percentage value to acquire NOX concentration.

SDS-PAGE. Protein samples were mixed with 4X loading buffer containing DTT and heated in a boiling water bath for 10 minutes, removed, and spun down on a bench top centrifuge. Samples were separated on a gel containing a 5% acrylamide stacking gel and a 15% acrylamide running or separating gel using a constant current of 35 milliamperes for approximately 1 hour. Gels were stained with Coomassie blue stain, rinsed with water, and destained. Images were taken on a UVP MultDoc-IT Digital Imaging System.

Size Exclusion Chromatography with Multiangle Light Scattering and Refractive Index Detection. Samples separated over a WTC-100S5 (Wyatt Technologies) size exclusion column utilizing an Agilent 1200 HPLC to apply and maintain a 0.7 mL/min flow rate of 50 mM phosphate, pH 7.2 buffer containing 100 mM Sodium Chloride and 200 ppm Sodium Azide. Samples of 25 μ L were injected onto the column and total run time was 30 minutes. Samples were detected using a UV-Vis detector (Agilent), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALS) and detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). The number average molecular weight, M_n , was calculated with Astra 5.3.14 software (Wyatt Technology Corporation) based on the molecular weight distribution. Concentrations of encapsulated NADH oxidase were determined as previously described^[1].

Transmission Electron Microscopy. Samples (5 μ L, 0.1 mg/mL protein) were applied to formvar coated grids and incubated for 30 seconds and excess liquid was wicked away with filter paper. Grids were then stained with 5 μ L 2.5% uranyl acetate and excess stain was wicked away with filter paper. Images were taken on a LEO 912AB transmission electron microscope at accelerating voltage of 100 kV.

Kinetic Assays. Turnover of P22-NOX was compared to free NADH oxidase (FO) in 50 mM phosphate, 25 mM NaCl pH 7.5, with and without 100 μ M flavin adenine dinucleotide (FAD). Three morphologies of the P22 capsid were assayed: procapsid, expanded shell, and wiffle ball. Expanded shell and wiffle ball morphologies were acquired by heating sample at 65 $^{\circ}$ C and 75 $^{\circ}$ C, respectively, for 20 minutes, spinning down on a table top centrifuge at 17 g for 5 minutes, and collecting supernatant. Activity assays were carried out on an Agilent 8453 UV-Vis Spectrophotometer fitted with an 8 position multicell transport utilizing a VWR temperature control module. All assays were conducted with a final assay volume of either 80 μ L or 100 μ L at 37, 50, 60, or 70 $^{\circ}$ C and performed in triplicate (excluding those morphologies that are temperature dependent (e.g. procapsid cannot be assayed above 55 $^{\circ}$ C or expanded shell will start to form)). Buffers were preheated in a quartz cuvette for 2 minutes prior to assay. NADH was added to each reaction so that $A_{340} = 1.6-2.0$. NADH thermal degradation was monitored for 100-200 seconds then enzyme (either 1, 2, or 3 μ L, depending on temperature) was mixed in solution. Reactions were mixed thoroughly by pipetting and enzyme activity was attributed to the conversion of NADH to NAD^+ as monitored by the depletion of the absorbance at 340 nm. The extinction coefficients of NADH, P22 coat protein, and NOX-SP were calculated to be 6.22 $mM^{-1}cm^{-1}$, 44.38 $mM^{-1}cm^{-1}$, and 27.98 $mM^{-1}cm^{-1}$, respectively (ExPASy's ProtParam tool). Turnover values were calculated from the maximum observed initial rate in each assay after thermal degradation was subtracted.

Hydrogen Peroxide Determination. Hydrogen peroxide formation was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and horseradish peroxidase (HP) as described previously^[3], with minor adjustments. Briefly, 10 μL of appropriate morphologies of P22-NOX and free NADH oxidase were run to completion in 50 mM NaPO_4 , 25 mM NaCl , 100 μM FAD pH 7.5 in either 37 $^\circ\text{C}$ or 70 $^\circ\text{C}$ water baths as monitored by NADH depletion before and after reactions (A_{340}). 200 μL of buffer was added to each reaction after NADH depletion was completed and samples were allowed to cool to room temperature (20-30 minutes). 150 μL of 22 $\text{mg}\cdot\text{mL}^{-1}$ ABTS 100 $\text{U}\cdot\text{mL}^{-1}$ HR was added to each sample and absorbance at 725nm was recorded after a 30 minute room temperature incubation period. The absorbance was then fitted to a standard curve to determine H_2O_2 concentration, and subsequently the equivalents of H_2O_2 produced per NADH.

Anaerobic Assay of P22-NADH oxidase. The ability of NADH oxidase to utilize H_2O_2 as a substrate was performed by monitoring NADH depletion under anaerobic conditions on an Agilent 8453 UV-Vis Spectrophotometer fitted with an 8 position multicell. 950 μL (cuvette A) or 945 μL (cuvette B) of 60 $\mu\text{g}\cdot\text{mL}^{-1}$ P22-NOX procapsid in standard buffer was degassed in an anaerobic cuvette with argon. 5 μL of degassed 10 mM H_2O_2 was added to cuvette B to give a final concentration of 50 μM (values consistent with natural enzyme production). 50-70 μL of 9 $\text{mg}\cdot\text{mL}^{-1}$ NADH was added to each cuvette and depletion was monitored at 340nm.

Bacteriocidal Properties. The capacity of encapsulated NADH oxidase to kill bacterial cultures was tested on a 96 well plate under varying conditions. Optical density was measured on a SPECTRA max 384 PLUS with SoftMax® 5 Pro software at A_{600} . All wells contained 135 μL LB. 0.45 μL aliquots of an overnight *E. coli* BL21 culture were added to three concentrations of P22-NOX procapsid (5.7, 4, and 2 mg/mL), one concentration of free NADH oxidase (2.2 mg/mL), and a well containing empty P22 procapsid. The positive control consisted of LB with bacterial culture while the negative control contained only LB and 15 μL PBS. 7.5 μL NADH was added to those wells that contained oxidase (encapsulated and free) along with an NADH control (135 μL LB, 0.45 μL culture). All experiments were performed in quadruplicate. A blank was taken immediately after mixing. Absorbance was measured every hour for 8 hours. The plate was shaken at 37 $^\circ\text{C}$ between absorbance measurements.

Immobilization Assay. P22-NOX (procapsid morphology) was immobilized on glass beads and tested for activity. 0.2 g of 0.5mm glass beads were treated with a 3:1 ratio of H_2SO_4 :30% H_2O_2 for 10 minutes with intermittent vortexing. 10% (3-Aminopropyl)triethoxysilane (APTES) in

ethanol was then added to the solution for 20 minutes with vortexing. Beads were washed 3x with ethanol then dried in a Blue M electric utility laboratory oven at 100°C. To functionalize carboxylate groups on protein, 2.2 mg N-hydroxysuccinimide (NHS) and 0.8 mg 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) were added per 1 mg of protein to the beads for 30 minutes with intermittent vortexing. A total of 200 µL of 1 mg/mL P22-NOX was functionalized. Amine functionalized beads were mixed with functionalized protein for 2 hours at room temperature with constant rocking and periodic vortexing. Beads were then washed 4x with buffer to remove free protein. H₂O₂ determination was completed as described above and reported qualitatively. After assay, beads were washed and stored in 200 µL buffer at 4°C. The assay was repeated one week later.

DNA Sequences

NOX-SP

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Free NOX

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K118C P22 Coat Protein

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Protein Sequences

NOX-SP

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GDVETRYRKLKAKLKGIR

Free NOX

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K118 P22 Coat Protein

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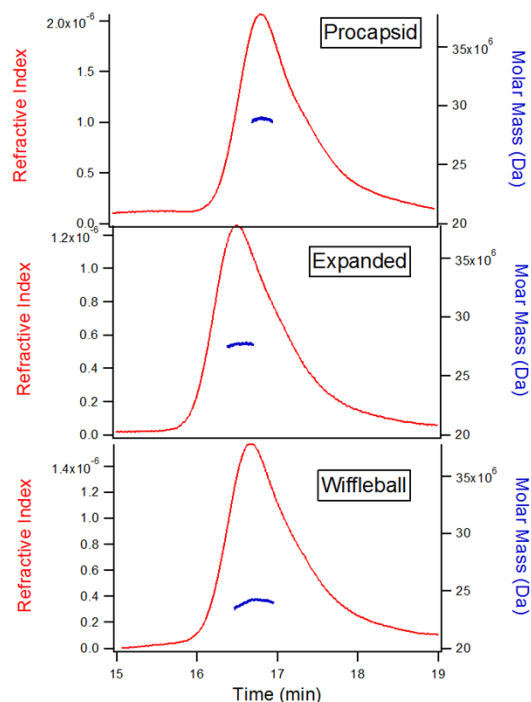
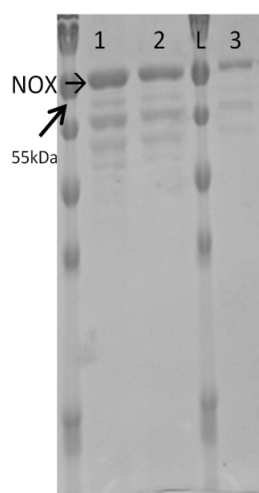


Figure S1. Characterization of NOX-P22 constructs by Size Exclusion Chromatography coupled to multi-angle light scattering and refractive index detectors (SEC-MALS/RI). Chromatograms of NOX-P22 PC, EX, and WB showing elution time, molecular weight distribution (blue), and refractive index detector trace (red).

Construct	M_N (MDa)	Mass Cargo (MDa)	R_g (nm)	R_h (nm)	R_g/R_h
PC NOX-SP	28.8 ± 0.60	9.2	24.0 ± 0.05	28.6 ± 0.09	0.84

P22					
EX NOX-SP P22	27.3 ± 0.49	7.2	26.8 ± 0.1	31.0 ± 0.3	0.86
WB NOX- SP P22	24.4 ± 0.27	7.6	26.2 ± 0.05	30.7 ± 0.09	0.85

Table S1. Data determined from SEC-MALS/RI characterization. Values given are for the averages of three measurements.



Lane number	Densitometry value for NOX band
1	62.9%
2	54.4%
3	48.5%
Average	55.3 ± 5.9%

Figure S2. Densitometry of free NOX. Three concentrations of free NOX were examined by SDS-PAGE. Densitometry values from each lane for the band corresponding to NOX were averaged. The free NOX concentration determined by BCA assay was multiplied by 0.553 to determine actual concentration.

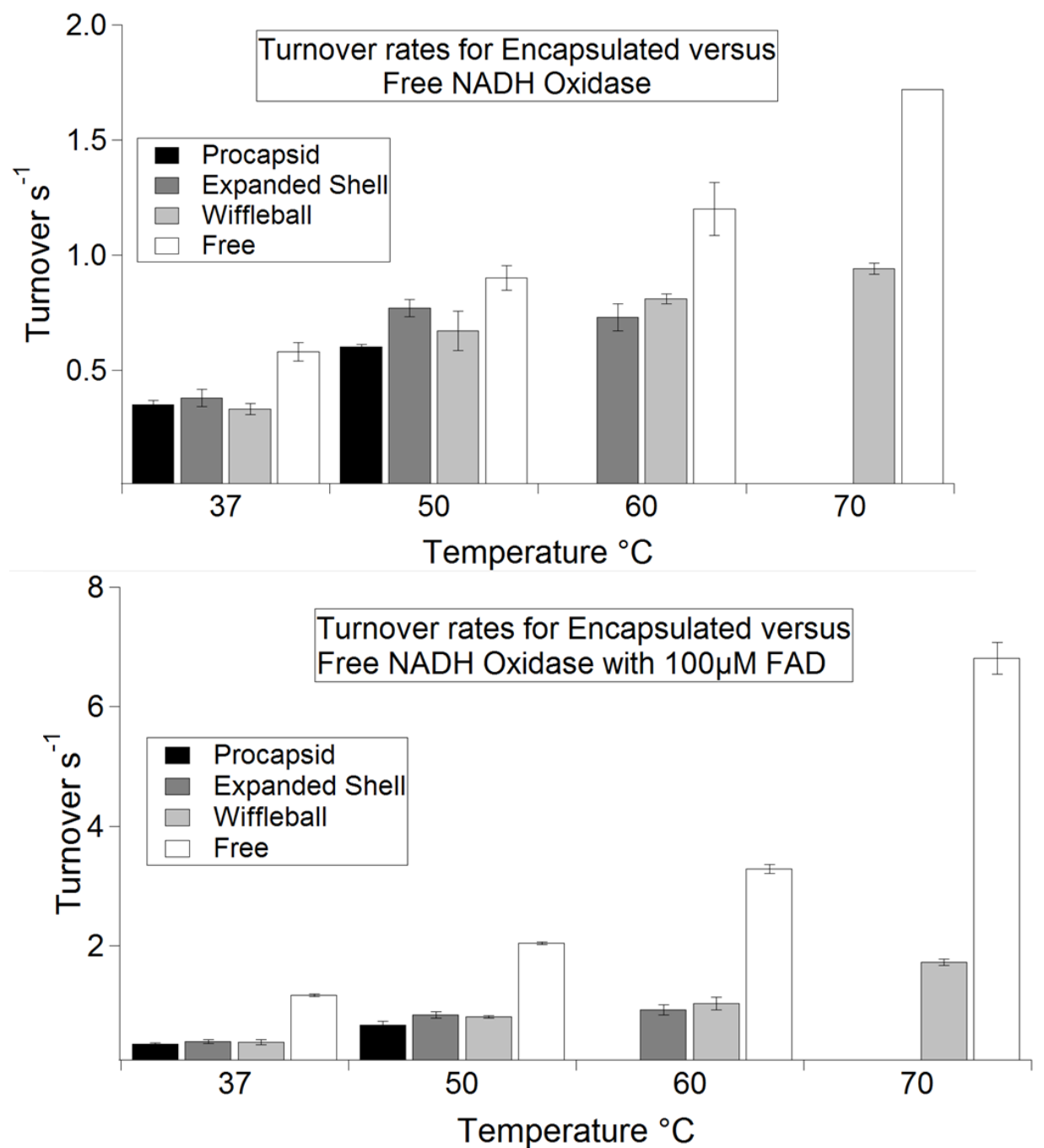


Figure S3. Turnover rates of P22-NOX PC, EX, and WB and free NOX without (above) and with (below) 100µM FAD.

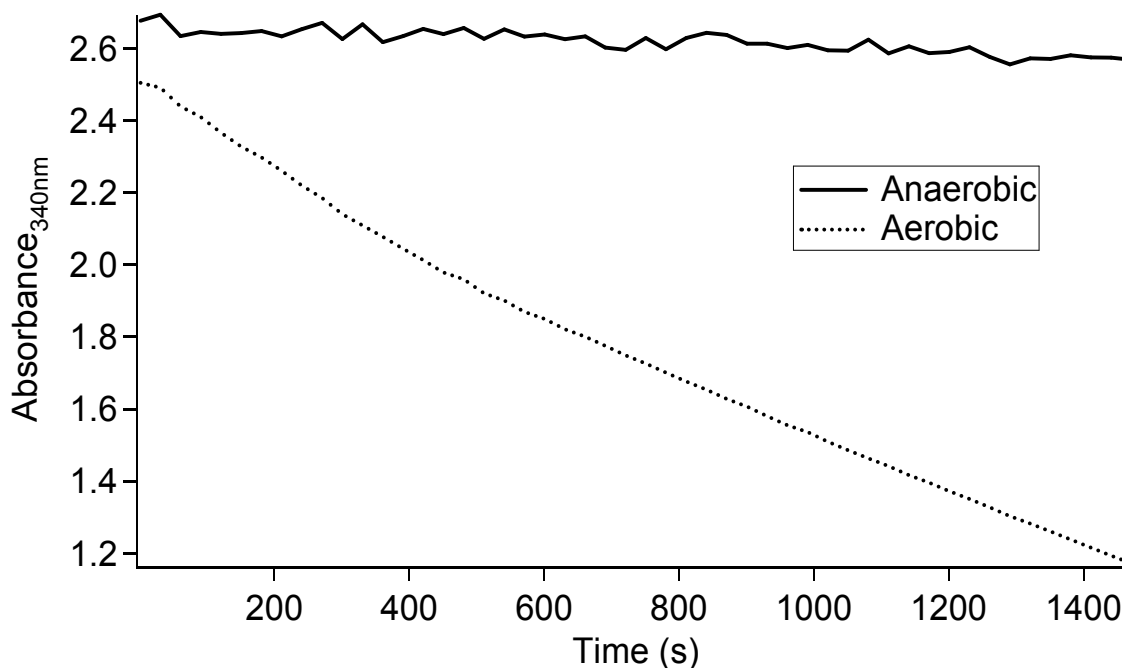


Figure S4. Substrate specificity of NOX. Anaerobic versus aerobic activity assay of P22-NOX. P22-NOX nanoparticles were tested for activity in an aerobic or anaerobic environment. The aerobic control (dashed lines) shows depletion of NADH while the anaerobic cuvette, supplemented with hydrogen peroxide, does not. This indicates that NOX does not utilize H₂O₂ as a substrate.

Table 2. Equivalents of Hydrogen peroxide produced per NADH by free and encapsulated NADH oxidase

Temp.	Sample	A _{340nm}	NADH concentration (μM)	A _{725nm}	H ₂ O ₂ concentration (μM)	Ratio of H ₂ O ₂ produced per NADH	Standard Deviation
37°C	Procasid	0.58	93.57	1.40	46.55	0.497	0.032
	Expanded Shell	0.59	95.64	1.38	45.79	0.479	0.001
	Wiffle Ball	0.60	95.86	1.42	47.08	0.491	0.003
	Free	0.59	95.08	1.41	46.89	0.493	0.004
70°C	Wiffle Ball	0.58	92.45	1.33	44.09	0.477	0.003
	Free	0.59	95.59	1.35	44.88	0.469	0.000

Table S2. Determination of the ratio of hydrogen peroxide made for every NADH cofactor consumed.



Figure S5. NOX-P22 immobilization on silica beads. P22-NOX was covalently attached to 0.5mm silica beads and assayed for activity 11 days after attachment. Encapsulated enzymes retained activity after immobilization.

- [1] D. P. Patterson, B. Schwarz, K. El-Boubbou, J. van der Oost, P. E. Prevelige, T. Douglas, *Soft Matter* **2012**, *8*, 10158-10166.
- [2] D. P. Patterson, P. E. Prevelige, T. Douglas, *Acs Nano* **2012**, *6*, 5000-5009.
- [3] K. K. Mäkinen, J. Tenovuo, *Analytical biochemistry* **1982**, *126*, 100-108.