Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Electronic Supplementary information

Cytochrome C with Peroxidase-like Activity Encapsulated Inside the Small DPS Protein Nanocage†

Hitesh Kumar Waghwani [a] and Trevor Douglas*[a]

[a] Department of Chemistry, Indiana University, 800 East Kirkwood Avenue, Bloomington, Indiana 47405, United States

* Corresponding author:
Trevor Douglas, E-mail: trevdoug@indiana.edu

Table of contents:

Section 1: Characterization of Dps C101S C126S (Dps)

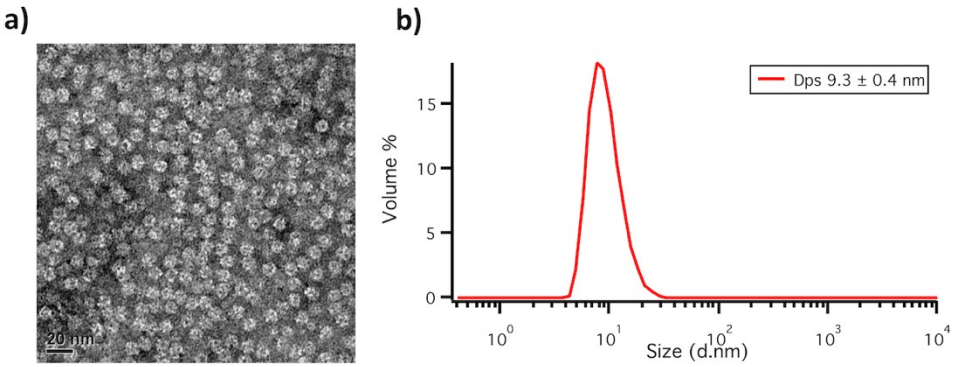

Figure S1. TEM and DLS characterization of cysteine engineered Dps (Dps C101S C126S) confirms formation of 9.3 $\pm$ 0.4 nm cage structure similar to wtDps.

Section 2: Characterization of 6His-Dps-CytC subunits assembly

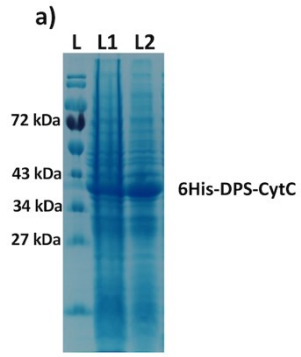

Figure S2. SDS-PAGE analysis of recombinant 6His-Dps-CytC fusion protein shows presence of protein in cell pellet (Lane L1) and in cell lysis pellet (Lane L2) as an insoluble aggregate suggesting 6His-Dps-CytC subunits failed to self-assemble into cage structure when overexpressed in *E. coli*.

Section 3: Supporting calculations

Volume calculations

$Dps_{interior}$: r =2.5 nm (PDB 2CLB)[1]

$Vol_{Dps} = \left(\frac{4}{3}\right) * 3.14 * (2.5)^3$ = 65.45 nm^3

CytC: r =1.75 nm

$Vol_{CytC} = \left(\frac{4}{3}\right) * 3.14 * (1.75)^3$ = 22.45 nm^3

Molar confinement and percent occupancy calculations

$$Mconf\ cargo = \frac{\frac{\#CytC\ per\ Dps}{6.022\ x\ 10^{23}\ Enzymes\ per\ mole} * \frac{1000\ mmol}{1\ mol}}{Volume\ of\ Dps\ interior}$$

$$Mconf\ DpsCytC = \frac{\frac{1\ CytC}{6.022\ x\ 10^{23}\ CytC\ per\ mole} * \frac{1000\ mmol}{1\ mol}}{65.45\ nm^3}$$ = 25.37 mM

Molar confinement for 1 CytC/Dps is 25.37 mM

Percent Occupancy Calculation

Example calculation for DpsCytC

$$\%\ Occupancy = \frac{(\#CytC\ enzyme\ x\ Vol\ CytC)}{Vol\ of\ Dps\ interior}$$ x 100

$$\%\ Occupancy = \frac{(1\ x\ 22.45\ nm^3)}{65.45\ nm^3}$$ x 100

$\%\ Occupancy =$ 34.30 % (1CytC per Dps)

Section 4: Characterization of DpsCytC chimeric cage

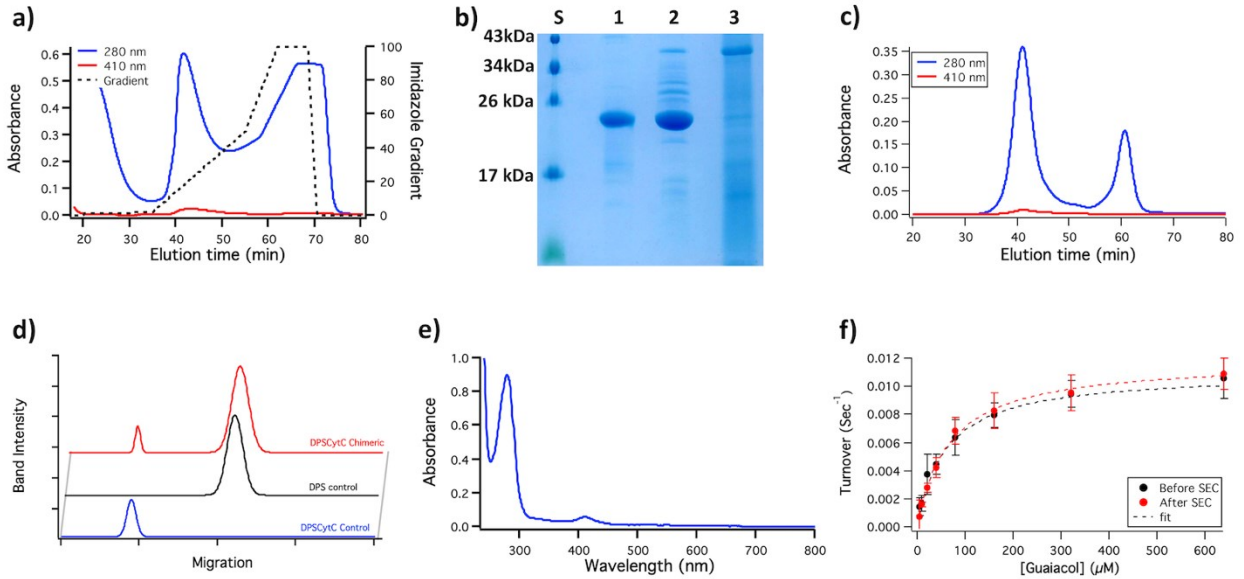

Figure S3. DpsCytC chimeric cage characterization. **a)** Ni-chelate affinity purification of His-tagged DpsCytC chimeric cages showing protein elution (blue) and heme elution (red) with imidazole gradient (10-500 mM, black trace). **b)** SDS-PAGE analysis showing the presence of both Dps and 6His-Dps-CytC protein subunit bands in chimeric cage sample (Lane 2). Lane S is standard protein ladder and Lane 1 and 3 are Dps subunits (Mw 21.7 kDa) and 6His-Dps-CytC subunits dissolved in 3M urea (Mw 36 kDa) as controls. **c)** DpsCytC chimeric cage SEC purification monitored at 280 nm and 410 nm for protein and heme peak respectively. **d)** Densitometry analysis of purified DpsCytC chimeric cages (from Figure S3b SDS-PAGE gel) with Dps and 6His-Dps-CytC subunits as controls. Line scan profile was fit with Gaussian multipeak fit function and calculated peak areas from it corresponded to 1:11 stoichiometry of 6His-Dps-CytC over Dps suggesting 1 CytC enzyme encapsulated per DpsCytC chimeric cage. **e)** UV-vis spectrophotometry on DpsCytC chimeric cages showing presence of protein and heme peak at 280 nm and 410 nm respectively. **f)** Plots of initial turnover against guaiacol concentration showing identical catalytic activity of DpsCytC at pH 6.25 before and after size exclusion purification at same heme concentration.

Table S1. Kinetic parameters for DpsCytC determined for guaiacol oxidation at pH 6.25 (before and after SEC purification)

Sample	kcat (Sec^{-1})	Km (μM)
Before SEC	0.020 ± 0.0004	53.43 ± 3.02
After SEC	0.020 ± 0.001	56.43 ± 8.53

Section 5: Passive encapsulation of rCytC inside Dps cage during self-assembly

To investigate the encapsulation of rCytC inside Dps during self-assembly, we performed three vector simultaneous co-expression of rCytC, Cytochrome C maturation gene and unmodified Dps in *E. coli* using similar culture growth conditions previously used for DpsCytC chimeric cage. Dps cages were purified by size exclusion chromatography followed by anion exchange chromatography and characterized for encapsulation of rCytC by SDS-PAGE (Fig. S4). Purified Dps sample showed negligible amount of rCytC encapsulation inside the Dps and was consistent with low heme:protein ratio of 0.0007 observed by UV-Vis spectroscopy. In contrast, the heme:protein ratio of chimeric assembled DpsCytC was observed to be 0.057 which is significantly higher and corresponds to encapsulation of a single heme matured CytC per ~5 Dps.

This experimental result confirms the limitations of a passive encapsulation approach (inability to purify selectively enzyme containing cages, poor experimental yields) and in contrast highlights the advantage of utilizing our chimeric self-assembly approach to selectively purify all enzyme containing cages etc.

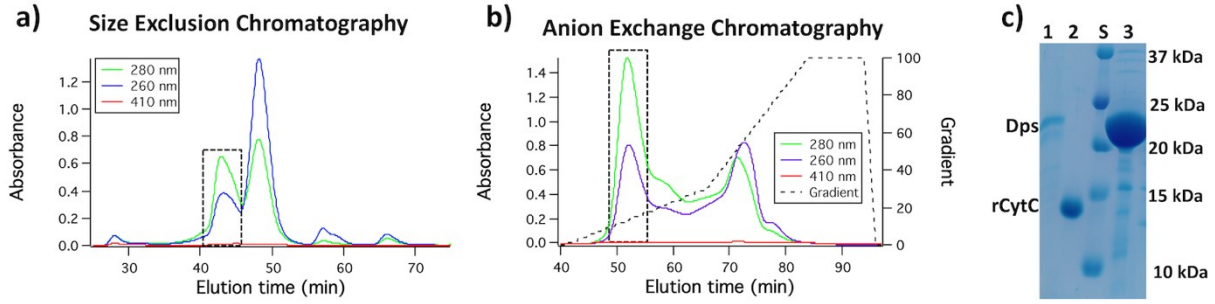

Figure S4. Purification and characterization of Dps passively encapsulating rCytC during assembly. **a)** Size exclusion chromatogram and **b)** Anion exchange chromatogram showing protein, nucleic acid and heme peak elution at 280, 260 and 410 nm respectively with inset showing Dps elution peak. **c)** SDS-PAGE gel analysis on anion exchange purified Dps showing very negligible amount of rCytC protein band (~12 kDa) along with high amount of Dps nanocage band.

Section 6: Characterization of rCytC

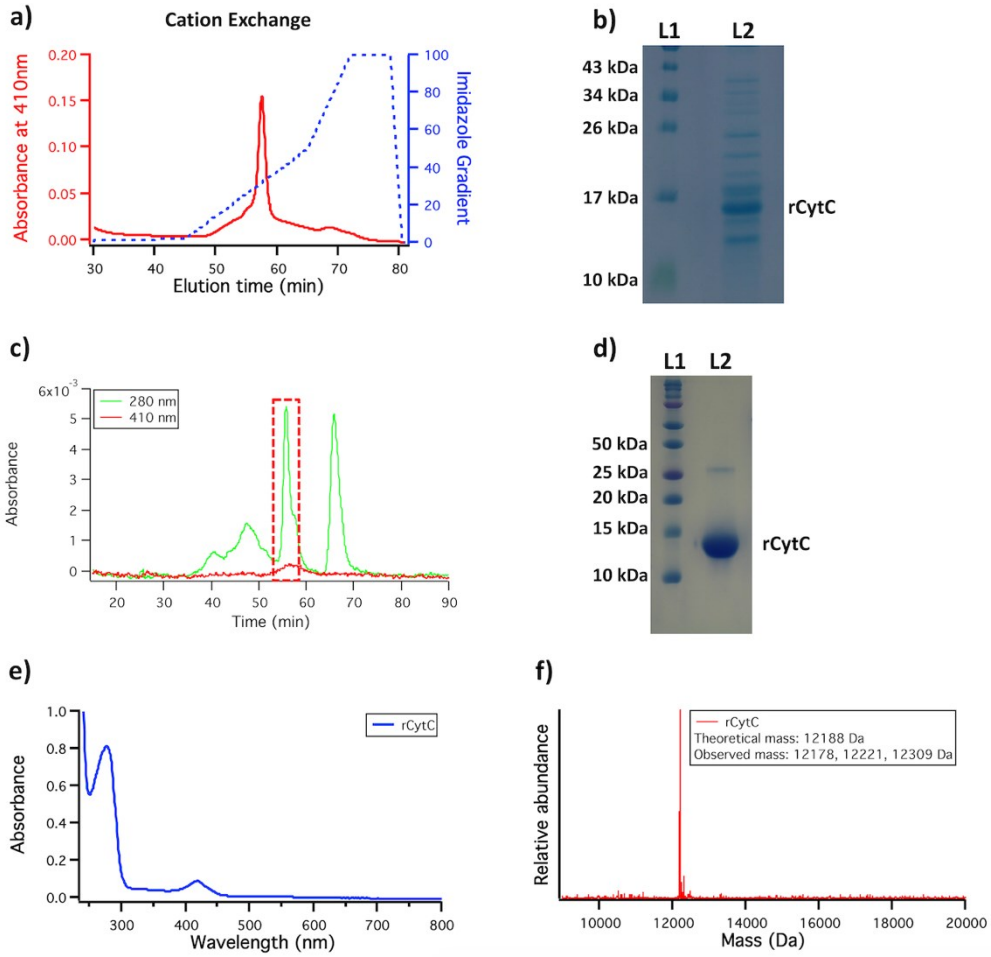

Figure S5. Characterization of free rCytC expressed in presence of CytC maturation gene. **a)** rCytC was purified by cation exchange column chromatography (HiTrap SP) using 0-1M NaCl gradient. **b)** SDS-PAGE analysis showed presence of rCytC protein as major band (Mw= 12.3 kDa) along with other protein impurities. **c)** Size exclusion chromatography was used to separate impurity proteins and obtain purified rCytC. **d)** SDS-PAGE analysis showing L1 as protein standard and L2 corresponding to SEC purified rCytC as major protein species. **e)** UV-Vis spectrophotometry on purified rCytC showed presence of protein at 280 nm and heme at 410 nm. **f)** Mass spectrometry

analysis of rCytC showing mass corresponding to rCytC (with methionine), without methionine and its acetylated species.

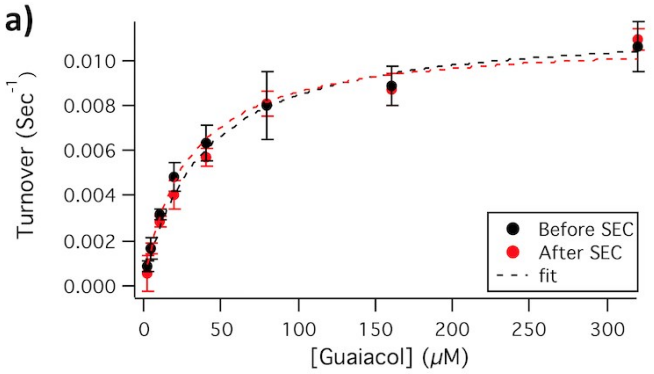

Figure S6. Plots of initial turnover against guaiacol concentration showing identical catalytic activity of rCytC at pH 6.25 before and after size exclusion purification at same heme concentration

Table S2. Kinetic parameters for rCytC determined for guaiacol oxidation at pH 6.25 (before and after SEC purification)

Sample	kcat (Sec^{-1})	Km (µM)
Before SEC	0.01 ± 0.0003	27.44 ± 2.8
After SEC	0.01 ± 0.0004	37.40 ± 5.0

Section 7: Characterization of commercial CytC (CytC_sigma)

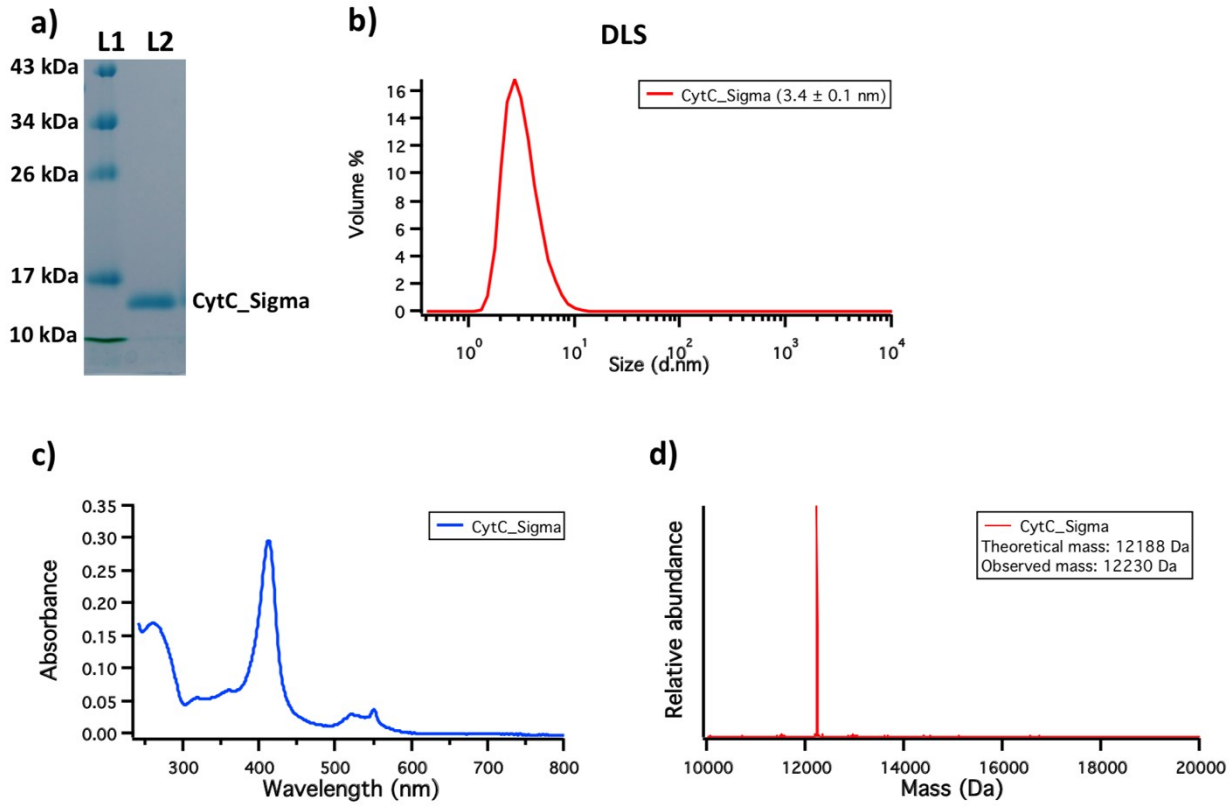

Figure S7. Characterization of CytC (purchased from sigma, CytC_sigma). **a)** SDS-PAGE gel showed single protein band corresponding to CytC (Mw= 12.3 kDa). **b)** DLS analysis showed 3.4 ±0.1 nm as diameter of CytC enzyme. **c)** UV-Vis spectrophotometry on CytC_sigma showed presence of heme peak at 410 nm. **d)** Mass spectrometry analysis of CytC_Sigma showing observed mass corresponding to acetylated CytC (without methionine).

Section 8: Stability of DpsCytC chimeric cage

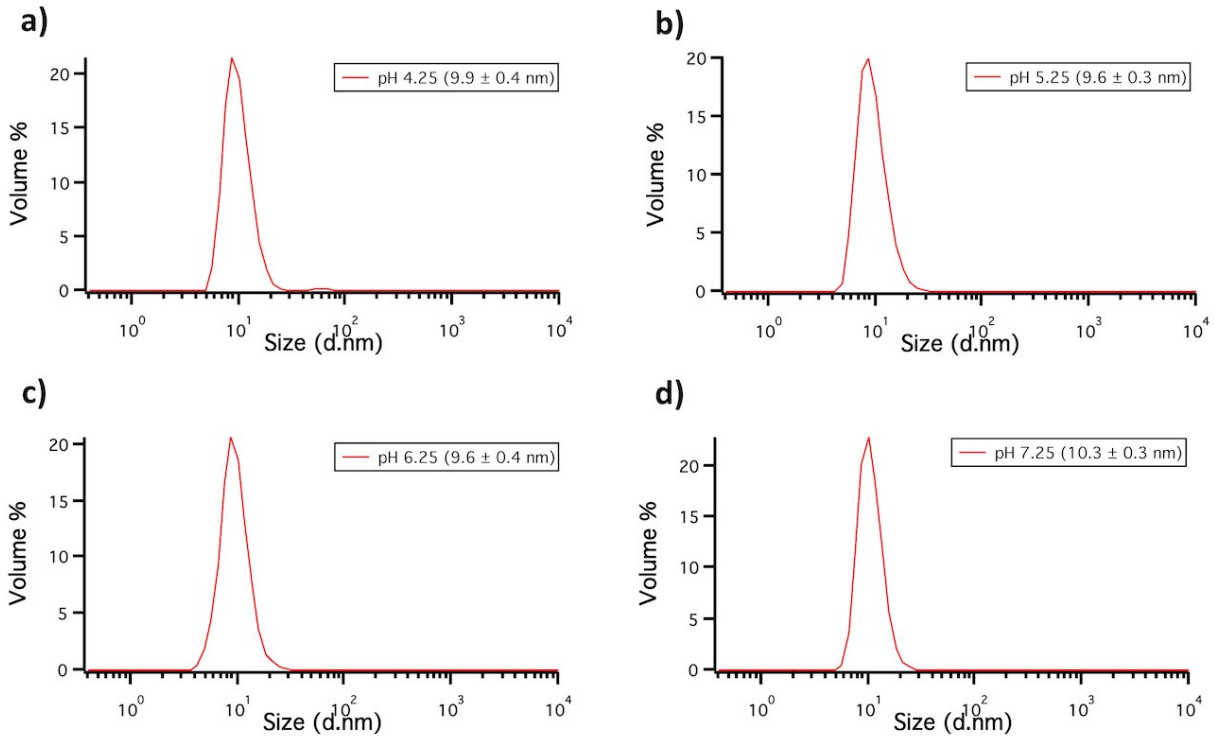

Figure S8. Stability of DpsCytC chimeric cages at various pH conditions was tested by DLS. Diameter of DpsCytC cages did not change upon incubation at varying pH conditions confirming pH stability of chimeric cage structure.

Section 9: rCytC vs CytC_sigma pH dependent peroxidase-like activity

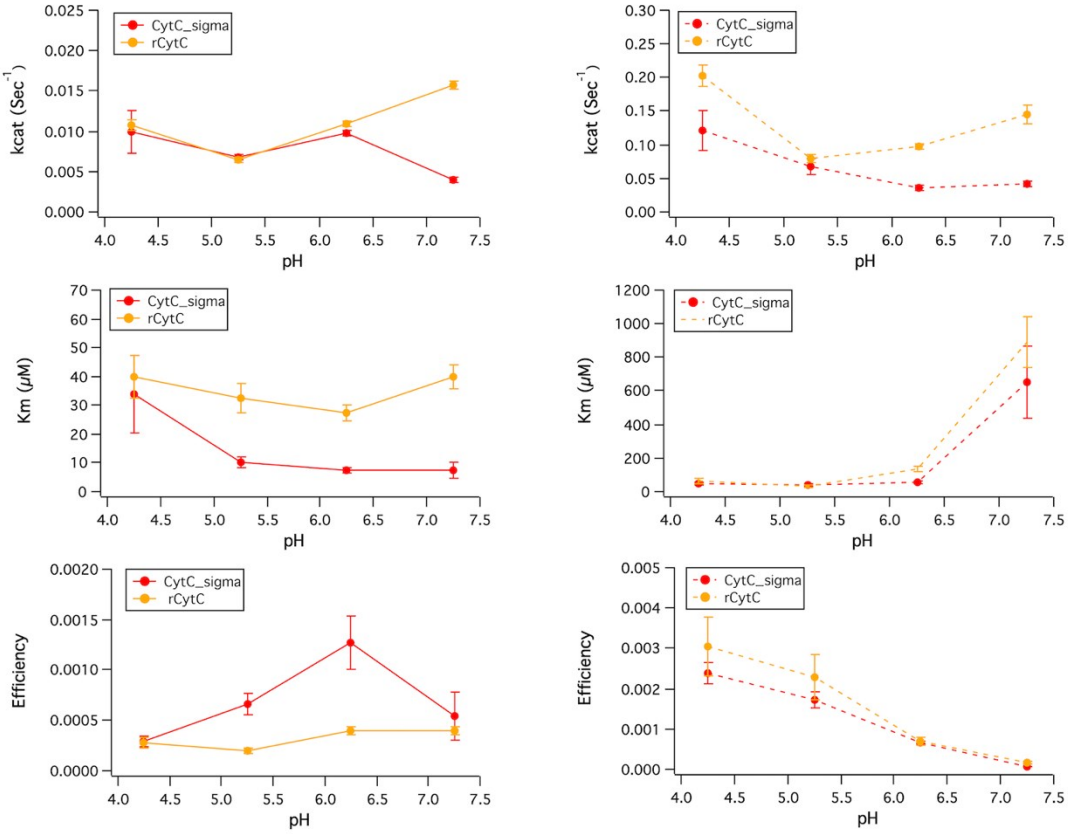

Figure S9. pH dependent peroxidase-like activity of rCytC and CytC_sigma. Panel on left showing catalytic activity comparison of rCytC (Orange trace) and CytC_ sigma (Red trace) for guaiacol oxidation whereas panel on right showing catalytic activity comparison for TMB oxidation.

Section 10: Catalytic activity initial turnover plots

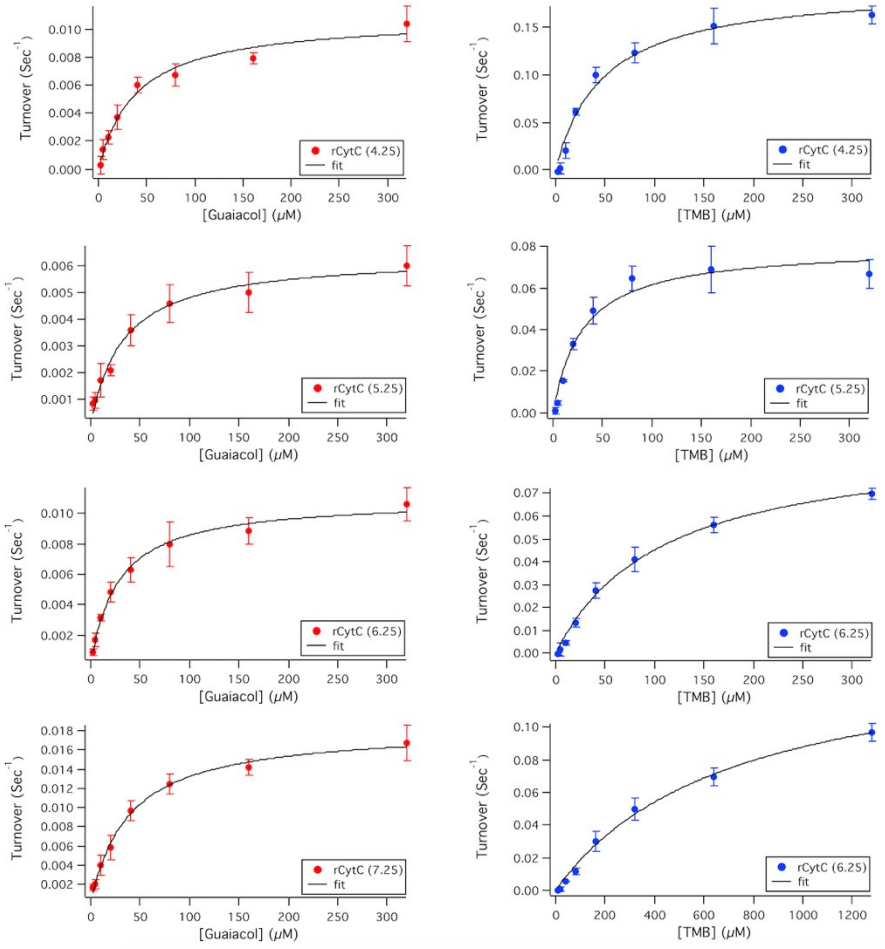

Figure S10. Plots of initial turnover against Guaiacol (left, in red) and TMB (right, in blue) concentration to assess pH dependent activity of rCytC.

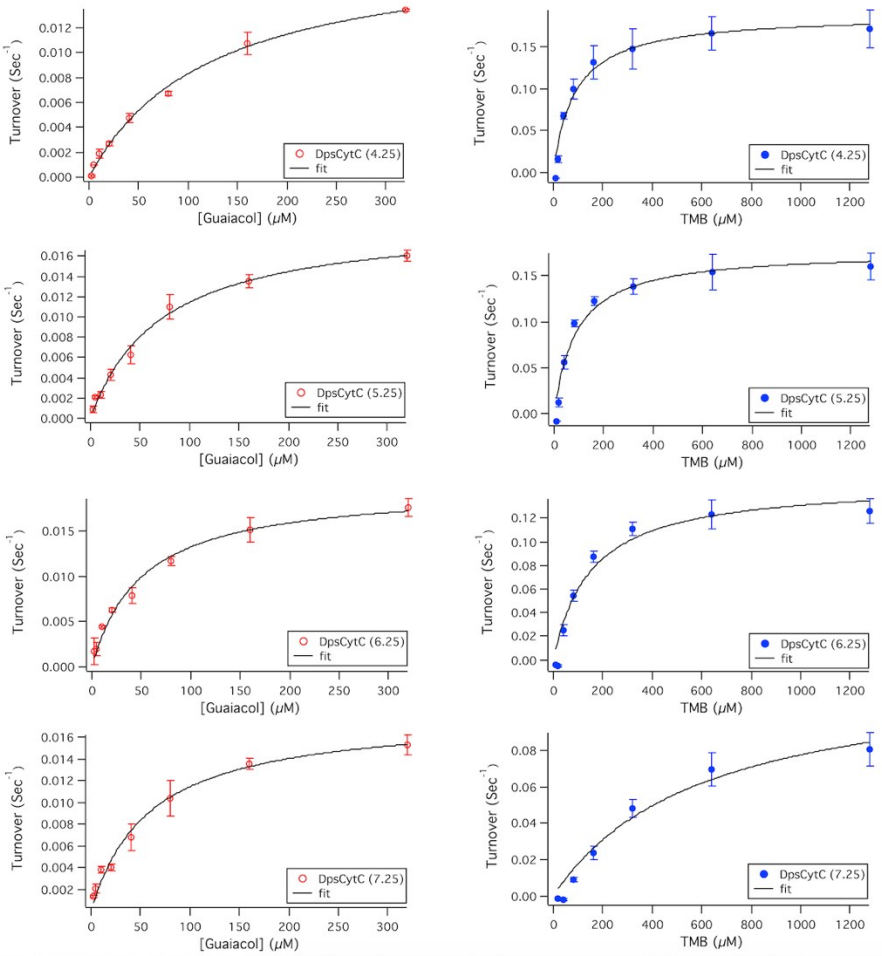

Figure S11. Plots of initial turnover against Guaiacol (left, in red) and TMB (right, in blue) concentration to assess pH dependent activity of DpsCytC.

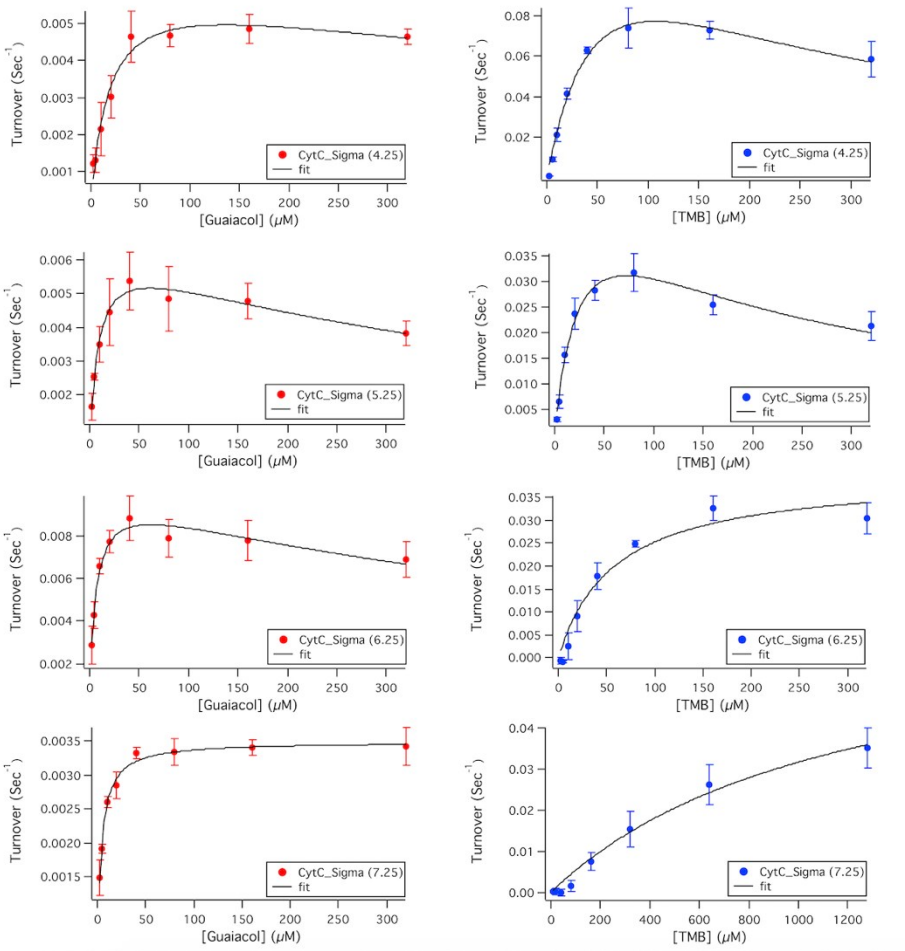

Figure S12. Plots of initial turnover against Guaiacol (left, in red) and TMB (right, in blue) concentration to assess pH dependent activity of CytC_Sigma.

Section 11: Substrate size calculation using SPARTAN software

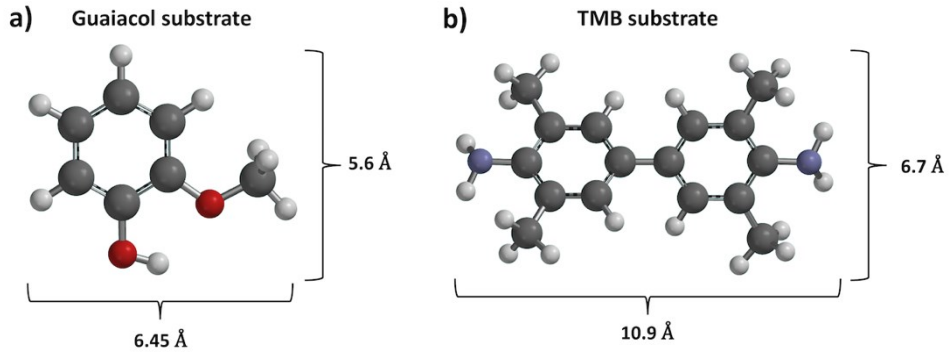

Figure S13. Structure of guaiacol and TMB substrate with substrate sizes (in angstroms) calculated using SPARTAN software (Wavefunction).[2] Atom-to-atom distance was taken into consideration while determining substrate size.

Section 12: Concentration dependent aggregation of free CytC

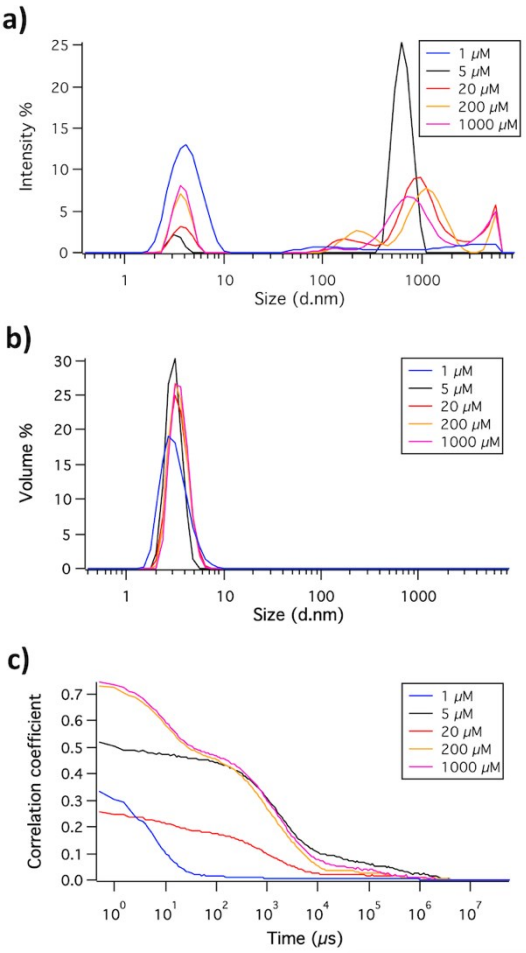

Figure S14. Measurement of free CytC aggregation behavior at varying concentrations by dynamic light scattering. a) Percent intensity plot; b) Percent volume plot; and c) Correlation coefficient plot respectively.

Table S3. Free CytC size distribution at different concentrations by percent intensity and volume respectively.

[CytC} µM	Intensity % Size (nm) Peak 1	Intensity % Size (nm) Peak 2	Intensity % Size (nm) Peak 3	Volume % Size (nm) Peak 1	Volume % Size (nm) Peak 2
1	4.4 ± 0.10	228 ± 210	3078 ± 290	3.2 ± 0.20	NA
5	3.3 ± 0.10	643 ± 53	NA	3.1 ± 0.10	682 ± 59
20	3.9 ± 0.20	754 ± 458	3808 ± 2226	3.5 ± 0.20	NA
200	3.8 ± 0.10	229 ± 56	1185 ± 226	3.5 ± 0.10	1490 ± 104
1000	3.8 ± 0.10	567 ± 369	3152 ± 2115	3.5 ± 0.10	809 ± 102

Section 13: Characterization of Dps-E158C conjugated with fluorescein

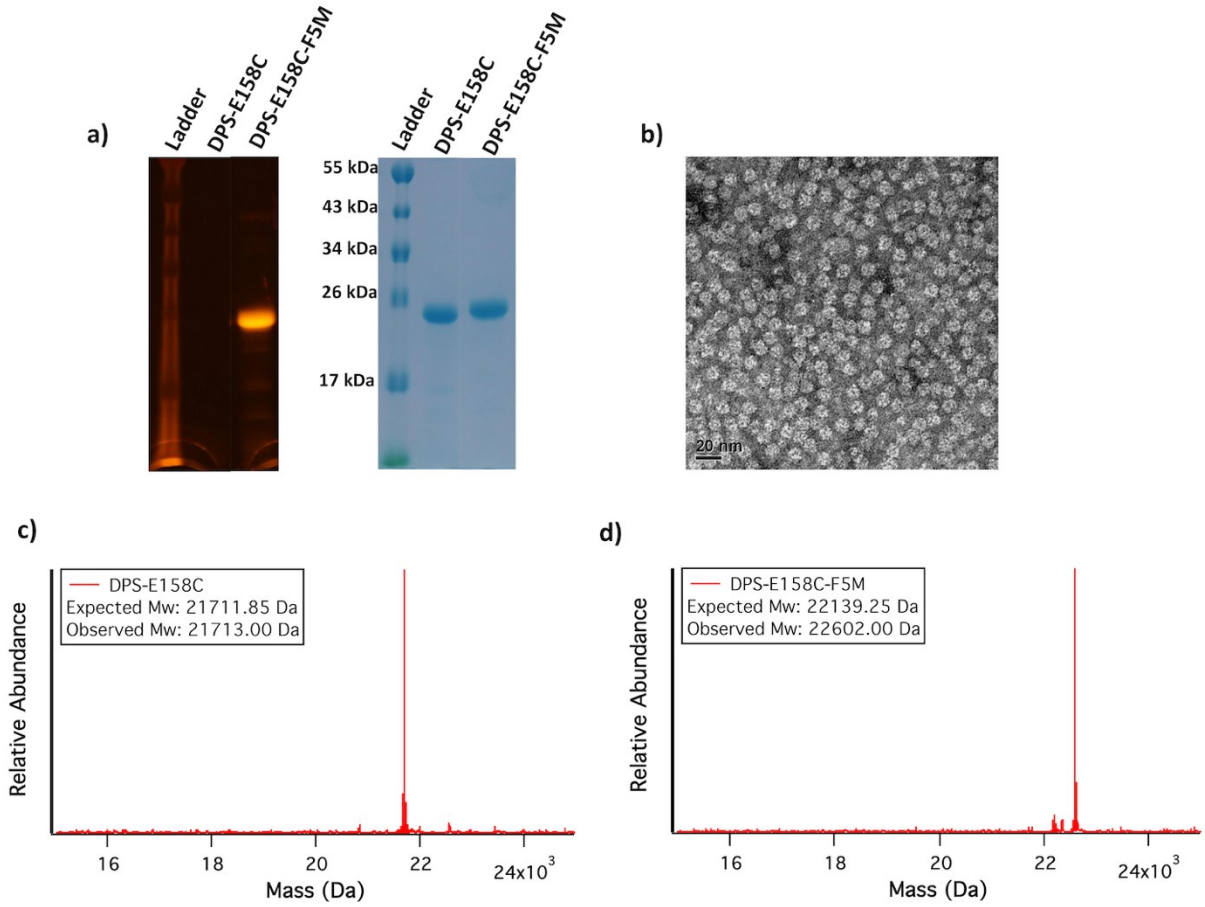

Figure S15. SDS-PAGE analysis of Dps-E158C before and after F5M labelling. **a)** The unstained gel illuminated with 488 nm laser and detected at 520 nm highlighted F5M labelling Dps-E158C and the coomassie blue stained gel showed presence of unlabeled and labelled Dps-E158C protein. **b)** TEM micrograph showing 9.5 ±0.3 nm Dps-E158C-F5M cages confirming maintenance of cage structure after fluorescein conjugation. **c)** Mass spectrometry characterization of Dps-E158C showing observed mass matched the expected mass for unlabeled protein whereas **d)** showing observed mass was higher than theoretical mass of 22139.25 Da or 22566.65 Da for single or double F5M labelled Dps-E158C protein respectively.

Section 14: pH dependent absorbance of Dps-E158C conjugated with fluorescein

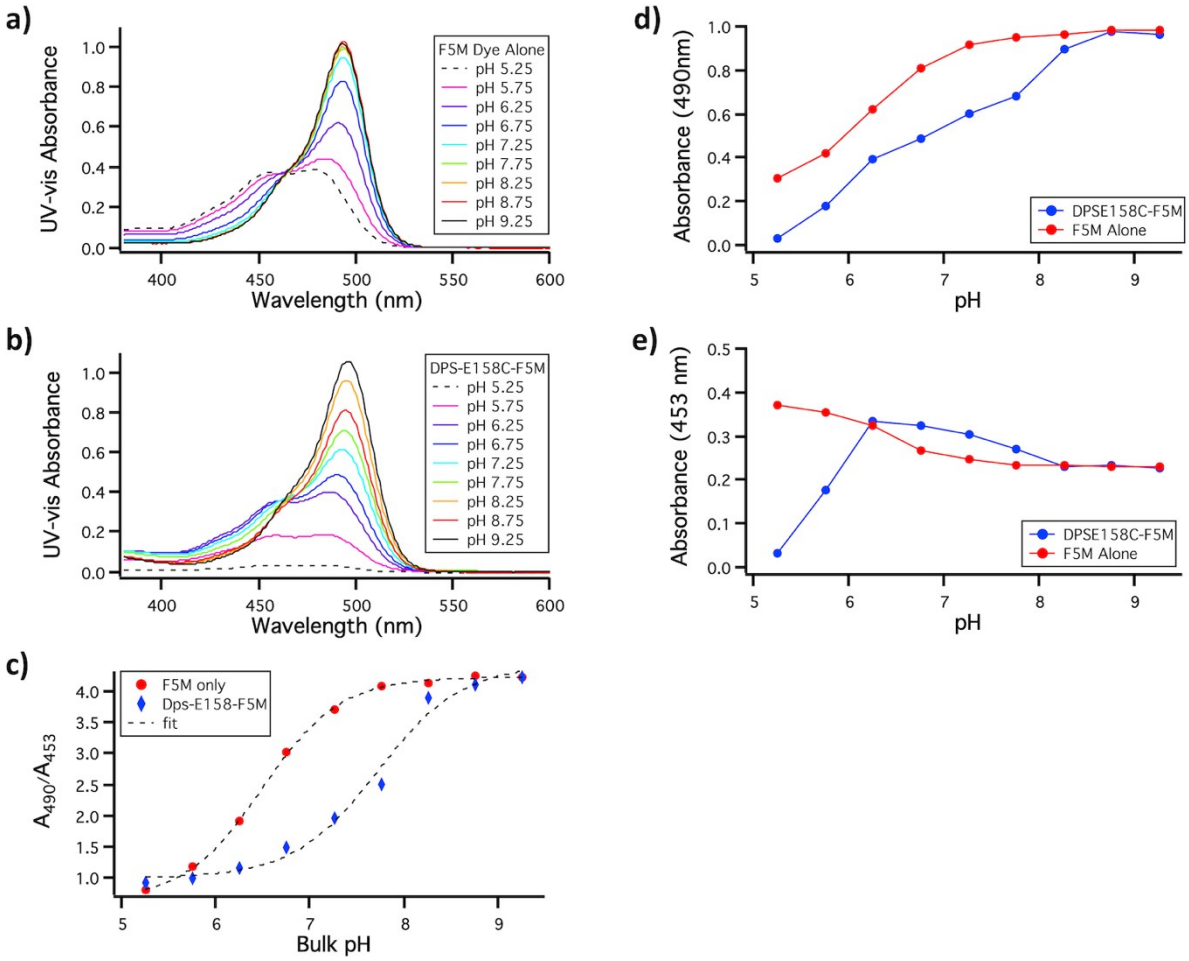

Figure S16. pH dependent UV-vis absorbance spectra of free and Dps-E158C conjugated F5M dye. **a & b)** pH dependent absorbance spectra of free F5M dye is significantly different compared to Dps-E158C conjugated F5M. **c)** Intensity plot obtained from ratio of absorbance of fluorescein dianion (A_{490}) over anion (A_{453}) at different bulk pH. **d & e)** pH dependent change in A_{490} and A_{453} for free F5M and conjugated F5M respectively.

Section 15: SYPRO Orange dye based thermal shift assay

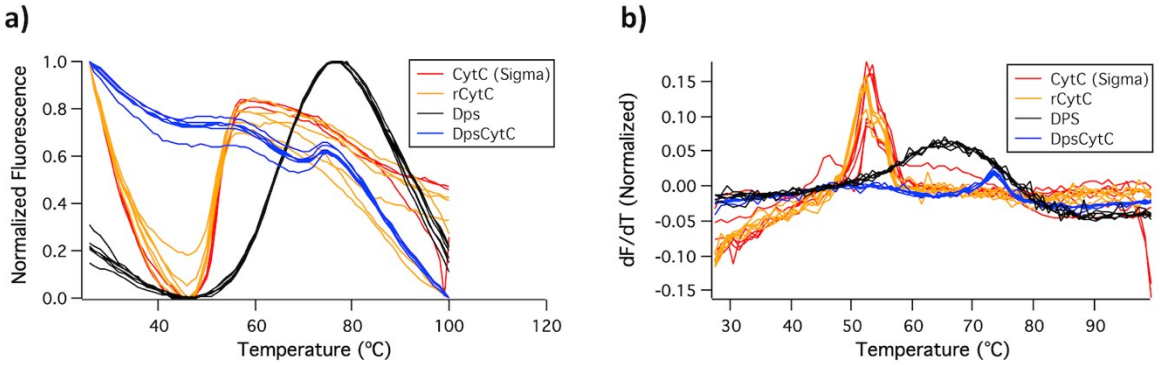

Figure S17. Thermal shift assay for characterizing thermal stability of Dps encapsulated CytC. **a)** Thermal denaturation profile. **b)** First derivative plot of thermal denaturation profile used to determine melting temperatures of free and encapsulated CytC. DpsCytC chimeric cage showed two stages of denaturation at ~52 °C and 73 °C respectively.

Section 16: Base pair sequence

Dps C101S C126S (Dps)

ATGCAAGAGAAACCCCAAGAACCAAAGGTAGTCGGAGTAGAAATCTTGGAAAAATCTGGGTTAGATAT
CAAAAAGTTAGTAGATAAACTAGTTAAAGCAACAGCTGCAGAGTTCACCACATATTACTACTATACCATA
TTGAGAATGCACCTAACTGGTATGGAAGGAGAGGGTCTTAAGGAAATAGCTGAGGACGCTAGGCTTGA
GGATAGACTTCACTTCGAACTAATGACTCAAAGGATTTATGAACTGGGAGGAGGCCTACCTAGAGACAT
AAGGCAGCTTGCTGATATTTCAGCTAGCTCCGATGCGTATTTACCAGAGAATTGGAAAGACCCTAAGGA
GATATTAAAGGTTTTGTTAGAAGCTGAGCAAAGCGCTATAAGAACTTGGAAAGAAGTATGTGATATGAC
GTATGGAAAAGATCCTAGAACTTACGATTTAGCGCAGAGGATACTTCAAGAAGAGATAGAGCACGAAG
CGTGGTTCTTAGAGTTACTATACGGAAGACCATCGGGTCATTTCAGAAGAAGTTCACCAGGGAACGCTC
CATATTCCAAGAAATAA

Dps C101S C126S E158C (Dps-E158C)

ATGCAAGAGAAACCCCAAGAACCAAAGGTAGTCGGAGTAGAAATCTTGGAAAAATCTGGGTTAGATAT
CAAAAAGTTAGTAGATAAACTAGTTAAAGCAACAGCTGCAGAGTTCACCACATATTACTACTATACCATA
TTGAGAATGCACCTAACTGGTATGGAAGGAGAGGGTCTTAAGGAAATAGCTGAGGACGCTAGGCTTGA
GGATAGACTTCACTTCGAACTAATGACTCAAAGGATTTATGAACTGGGAGGAGGCCTACCTAGAGACAT
AAGGCAGCTTGCTGATATTTCAGCTAGCTCCGATGCGTATTTACCAGAGAATTGGAAAGACCCTAAGGA
GATATTAAAGGTTTTGTTAGAAGCTGAGCAAAGCGCTATAAGAACTTGGAAAGAAGTATGTGATATGAC
GTATGGAAAAGATCCTAGAACTTACGATTTAGCGCAGAGGATACTTCAAGAAGAGATATGCCACGAAGC
GTGGTTCTTAGAGTTACTATACGGAAGACCATCGGGTCATTTCAGAAGAAGTTCACCAGGGAACGCTCC
ATATTCCAAGAAATAA

E158C mutation highlighted in red

Free CytC expressed in *E. coli* (rCytC)

ATGGGCGATGTCGAGAAAGGTAAGAAGATTTTCGTACAAAAATGTGCACAGTGTCACACTGTAGAGAA
AGGAGGCAAGCATAAGACTGGCCCCAATTTACATGGCTTATTCGGTCGTAAGACAGGCCAGGCCCCTGG
CTTCTCTTATACTGATGCAAATAAAAACAAGGGTATCACGTGGGGGGAAGAAACTTTAATGGAATATCT
TGAAAATCCGAAGAAGTACATCCCAGGGACCAAGATGATTTTCGCGGGGATTAAGAAAAAAGGTGAAC
GTGAGGACCTGATTGCGTATCTGAAAAAGGCCACAAACGAGTAA

6His-Dps-CytC fusion

ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGATGCAAGAGAAACCCCAAGAACCAAAGGTAGT
CGGAGTAGAAATCTTGGAAAAATCTGGGTTAGATATCAAAAAGTTAGTAGATAAACTAGTTAAAGCAAC
AGCTGCAGAGTTCACCACATATTACTACTATACCATATTGAGAATGCACCTAACTGGTATGGAAGGAGA
GGGTCTTAAGGAAATAGCTGAGGACGCTAGGCTTGAGGATAGACTTCACTTCGAACTAATGACTCAAAG
GATTTATGAACTGGGAGGAGGCCTACCTAGAGACATAAGGCAGCTTGCTGATATTTCAGCTAGCTCCGA
TGCGTATTTACCAGAGAATTGGAAAGACCCTAAGGAGATATTAAAGGTTTTGTTAGAAGCTGAGCAAAG
CGCTATAAGAACTTGGAAAGAAGTATGTGATATGACGTATGGAAAAGATCCTAGAACTTACGATTTAGC
GCAGAGGATACTTCAAGAAGAGATAGAGCACGAAGCGTGGTTCTTAGAGTTACTATACGGAAGACCAT
CGGGTCATTTCAGAAGAAGTTCACCAGGGAACGCTCCATATTCCAAGAAAGGCGCGGCAGGCGAAAAC
CTGTATTTCCAGAGCGGTGCGGCAGGCGGCGATGTAGAGAAGGGTAAAAAGATTTTCGTGCAAAAGTG
TGCGCAGTGCCATACCGTAGAGAAGGGCGGGAAACACAAGACTGGTCCTAATTTGCATGGCCTTTTTGG

GCGTAAGACAGGTCAGGCCCCTGGTTTCAGTTATACCGATGCCAACAAGAATAAAGGCATTACCTGGGG
GGAAGAAACTTTGATGGAGTATCTTGAGAATCCTAAGAAGTACATACCGGGAACGAAGATGATTTTCGC
GGGGATAAAGAAAAAGGGTGAACGTGAGGACTTGATTGCGTACTTAAAGAAGGCCACCAACGAGTAA

Section 17: Amino acid sequence

Dps C101S C126S (Dps)
MQEKPQEPKVVGVEILEKSGLDIKKLVDKLVKATAAEFTTYYYYTILRMHLTGMEGEGLKEIAEDARLEDRLHF
ELMTQRIYELGGGLPRDIRQLADISASSDAYLPENWKDPKEILKVLLEAEQSAIRTWKEVCDMTYGKDPRTYD
LAQRILQEEIEHEAWFLELLYGRPSGHFRRSSPGNAPYSKK

Dps C101S C126S E158C (Dps-E158C)
MQEKPQEPKVVGVEILEKSGLDIKKLVDKLVKATAAEFTTYYYYTILRMHLTGMEGEGLKEIAEDARLEDRLHF
ELMTQRIYELGGGLPRDIRQLADISASSDAYLPENWKDPKEILKVLLEAEQSAIRTWKEVCDMTYGKDPRTYD
LAQRILQEEICHEAWFLELLYGRPSGHFRRSSPGNAPYSKK
E158C mutation highlighted in red

Free CytC expressed in *E. coli* (rCytC)
MGDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLE
NPKKYIPGTKMIFAGIKKKGEREDLIAYLKKATNE

6His-Dps-CytC fusion
MGSSHHHHHHSQMQEKPQEPKVVGVEILEKSGLDIKKLVDKLVKATAAEFTTYYYYTILRMHLTGMEGEGLK
EIAEDARLEDRLHFELMTQRIYELGGGLPRDIRQLADISASSDAYLPENWKDPKEILKVLLEAEQSAIRTWKEVC
DMTYGKDPRTYDLAQRILQEEIEHEAWFLELLYGRPSGHFRRSSPGNAPYSKKGAAGENLYFQSGAAGGDVE
KGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKNKGITWGEETLMEYLENPKKYI
PGTKMIFAGIKKKGEREDLIAYLKKATNE
Linker with TEV protease recognition site underlined

Section 18: Molecular cloning

6His-Dps-CytC fusion protein

Primers for pCDFDuet-1 vector linearization
Fw Primer: gatccgaattcgagctcggcg
RV Primer: ctggctgtggtgatgatggtgatg

Plasmid linearization conditions: 95°C 2min, 95°C 20sec, 57°C 10sec, 70°C 2min.
1.5uL Fw primer, 1.5uL Rv primer, 1 uL pCDFDuet-1 empty vector as DNA template, 25 uL KOD mastermix, 21 uL dd water

G-block
The start and stop codons are highlighted in yellow, sequences that are complementary with pCDFDuet-1 vector are labeled in green, Dps is shown in blue, Linker with TEV protease site is underlined and CytC shown in red.

ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGATGCAAGAGAAACCCCAAGAACCAAAGGTAGT
CGGAGTAGAAATCTTGGAAAAATCTGGGTTAGATATCAAAAAGTTAGTAGATAAACTAGTTAAAGCAAC
AGCTGCAGAGTTCACCACATATTACTACTATACCATATTGAGAATGCACCTAACTGGTATGGAAGGAGA
GGGTCTTAAGGAAATAGCTGAGGACGCTAGGCTTGAGGATAGACTTCACTTCGAACTAATGACTCAAAG
GATTTATGAACTGGGAGGAGGCCTACCTAGAGACATAAGGCAGCTTGCTGATATTTCAGCTAGCTCCGA
TGCGTATTTACCAGAGAATTGGAAAGACCCTAAGGAGATATTAAAGGTTTTGTTAGAAGCTGAGCAAAG
CGCTATAAGAACTTGGAAAGAAGTATGTGATATGACGTATGGAAAAGATCCTAGAACTTACGATTTAGC
GCAGAGGATACTTCAAGAAGAGATAGAGCACGAAGCGTGGTTCTTAGAGTTACTATACGGAAGACCAT
CGGGTCATTTCAGAAGAAGTTCACCAGGGAACGCTCCATATTCCAAGAAAGGCGCGGCAGGCGAAAAC
CTGTATTTCCAGAGCGGTGCGGCAGGCGGCGATGTAGAGAAGGGTAAAAAGATTTTCGTGCAAAAGTG
TGCGCAGTGCCATACCGTAGAGAAGGGCGGGAAACACAAGACTGGTCCTAATTTGCATGGCCTTTTTGG
GCGTAAGACAGGTCAGGCCCCTGGTTTCAGTTATACCGATGCCAACAAGAATAAAGGCATTACCTGGGG
GGAAGAAACTTTGATGGAGTATCTTGAGAATCCTAAGAAGTACATACCGGGAACGAAGATGATTTTCGC
GGGGATAAAGAAAAAGGGTGAACGTGAGGACTTGATTGCGTACTTAAAGAAGGCCACCAACGAGTAA
GATCCGAATTCGAGCTCGGCGCGCCTGCAG

Section 19: References

1. G. H. Gauss, P. Benas, B. Wiedenheft, M. Young, T. Douglas and C. M. Lawrence, *Biochemistry*, 2006, **45**, 10815-10827.
2. Y. Shao, L. F. Molnar, Y. Jung, J. Kussmann, C. Ochsenfeld, S. T. Brown, A. T. B. Gilbert, L. V. Slipchenko, S. V. Levchenko, D. P. O'Neill, R. A. DiStasio, R. C. Lochan, T. Wang, G. J. O. Beran, N. A. Besley, J. M. Herbert, C. Y. Lin, T. Van Voorhis, S. H. Chien, A. Sodt, R. P. Steele, V. A. Rassolov, P. E. Maslen, P. P. Korambath, R. D. Adamson, B. Austin, J. Baker, E. F. C. Byrd, H. Dachsel, R. J. Doerksen, A. Dreuw, B. D. Dunietz, A. D. Dutoi, T. R. Furlani, S. R. Gwaltney, A. Heyden, S. Hirata, C. P. Hsu, G. Kedziora, R. Z. Khalliulin, P. Klunzinger, A. M. Lee, M. S. Lee, W. Liang, I. Lotan, N. Nair, B. Peters, E. I. Proynov, P. A. Pieniazek, Y. M. Rhee, J. Ritchie, E. Rosta, C. D. Sherrill, A. C. Simmonett, J. E. Subotnik, H. L. Woodcock, W. Zhang, A. T. Bell, A. K. Chakraborty, D. M. Chipman, F. J. Keil, A. Warshel, W. J. Hehre, H. F. Schaefer, J. Kong, A. I. Krylov, P. M. W. Gill and M. Head-Gordon, *Phys. Chem. Chem. Phys.*, 2006, **8**, 3172-3191.