Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary information

Induced Polymersome Formation from a Diblock PS-*b*-PAA Polymer via Encapsulation of Positively Charged Proteins and Peptides

David Hvasanov,[a] Jörg Wiedenmann,[b] Filip Braet[c] and Pall Thordarson*[a]

[a]*School of Chemistry, The University of New South Wales, Sydney, NSW, 2052, Australia. E-mail: p.thordarson@unsw.edu.au*

[b]*National Oceanography Center, University of Southampton, Southampton, SO14 3ZH, United Kingdom.*

[c]*Australian Centre for Microscopy & Microanalysis, The University of Sydney, Sydney, NSW, 2006, Australia.*

Contents:

Experimental

Materials. Yeast cytochrome *c* from *Saccharomyces cerevisiae* (catalog number C2436), calmodulin from bovine testes (catalog number P1431), bovine serum albumin (catalog number A0281), myoglobin from equine skeletal muscle (catalog number M0630), poly-*L*-lysine hydrobromide (1000-5000 Da, catalog number P0879) were purchased from Sigma Aldrich. Yeast iso-1 cytochrome *c* was purified following previously published procedures.[1] Oregon Green® 488 maleimide was purchased from Invitrogen. THF was obtained from a Pure Solv dry solvent system (Innovative Technology, Inc. model #PS-MD-7) and was filtered through a 0.45 μm cellulose membrane filter (Minisart RC 25, Sartonius Stedim Biotech) prior to polymer aggregation studies. For the aggregation studies and preparation of all salt buffers, ultra pure water ($R > 18\times10^6$ Ω) was used. Diblock copolymer, polystyrene$_{140}$-*b*-poly(acrylic acid)$_{48}$ (PDI = 1.10), was purchased from Encapson (The Netherlands, catalog number 1036). All aqueous buffers were pH adjusted with aqueous sodium hydroxide (1 M) or hydrochloric acid (1 M) using a Scholar 425 pH meter (Corning). All other chemicals were used as received.

Instrumentation. A Varian Cary 50 Bio UV-Vis or Cary 5 UV-Vis-NIR spectrometer was used for UV-Vis spectra measurements. Fluorescence spectra were recorded using a Varian Cary Eclipse spectrometer. TEM micrographs were recorded on a JEOL 1400 (80 kV) instrument and *cryo*-TEM micrographs were recorded on a JEOL 2100 (200 kV) instrument. Optical and confocal laser microscopy experiments were carried out with an Olympus Fluoview FV1000, fitted with monochromatic laser light sources for fluorescence measurements.

Preparation of PS-*b*-PAA polymersomes for encapsulation.

In a typical experiment, 33 μl of a 1 mg mL^{-1} solution of PS-*b*-PAA in THF was injected into 200 μL of a 5 μM enzyme solution in sodium dihydrogen phosphate buffer (20 mM, pH 7.0) or phosphate buffered saline (150 mM, pH 7.2). The solution was allowed to equilibrate for at least 24 h and extensively dialysed against water (2×1 L) using a 50 kDa molecular weight cut-off membrane over 24 h to remove non-encapsulated enzymes.

Transmission electron microscopy (TEM) studies.

The samples were prepared by placing 20 μL of sample onto a formvar-coated copper grid and the excess water was blotted away after 2 min. with a filter paper. For statistical analysis, a population of 100 micelles or polymersomes was measured from TEM micrographs for determination of the average and standard deviation of diameters. For analysis of polymersome-to-micelle (p/m) ratio, a population of 100 aggregates from TEM micrographs was selected and aggregate morphologies displaying distinct contrast within individual particles (indicating the presence of bilayer and cavity formation) were classified as vesicles whereas micelles were defined as particles showing uniform contrast. Ratio measurements were repeated in triplicate.

For *cryo*-TEM, 3 μL of sample was directly placed onto glow-discharged holey carbon grids (Quantifoil, Germany). Grids were blotted once at a blotting angle of 2 mm for 2 s under 100% relative humidity at 25 ^{o}C and subsequently plunged into liquid ethane using the automated vitrobot (F.E.I, The Netherlands). Vitrified samples were stored in liquid nitrogen upon cryo transfer for *cryo*-TEM investigation.[2,3]

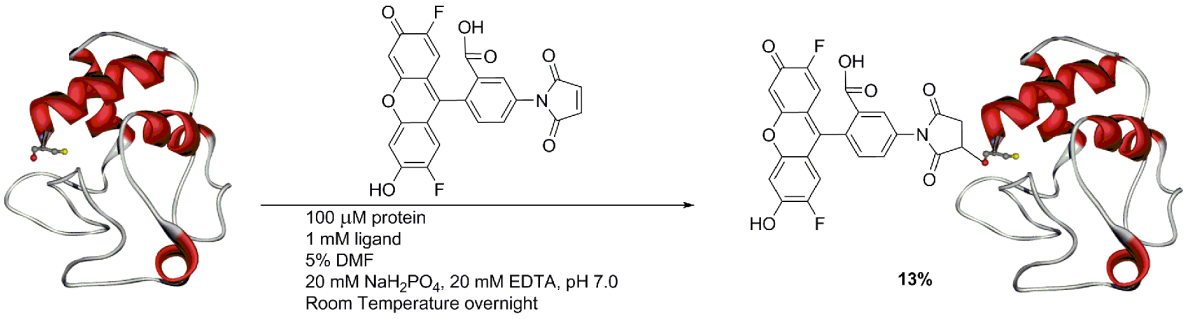
Confocal laser scanning microscopy studies.

Images were acquired on a confocal laser scanning microscope with a 40x 0.9NA water-immersion objective. Excitation was at 488 nm using an Ar^{+} laser. Detection was in the range 500-600 nm using internal PMTs (gains were set to 468 V). The confocal pinhole (aperture) was set to auto and images with a 640×640 pixel resolution were recorded at a scan rate of 40 μs/pixel with a total acquisition time of ≈ 16 s.

amGFP (amFP497) (*Acropora millepora*) expression and isolation

The coding sequence of the green fluorescent protein (GFP)-like protein from the reef coral *Acropora millepora* (amFP497)[4] was introduced in the plasmid pQE32 (Qiagen, Hilden, Germany), resulting in the addition a N-terminal 6 × histidine tag to the recombinant protein. Bacteria (*Escherichia coli*, M15 pREP4) were transformed with the plasmid and grown at 37 ºC in 2YT medium to an optical density of 0.6. Expression of the protein was induced with isopropyl-β-D-thiogalactopyranosid (IPTG) and the culture was incubated at 20 ºC on a shaker at 220 rpm for 12 h. Cultures were slightly agitated further for 7 days at 4 ºC to increase the yield of soluble protein.[5] Subsequently, cells were harvested and the recombinant protein was purified by immobilized metal ion chromatography using Talon™ matrix (Clontech; Palo Alto, USA), following the protocol of the manufacturer.

Synthesis of fluorescently labelled cytochrome *c*.



A solution of Oregon Green® 488 maleimide (0.46 mg, 9.9 × 10^{-4} mmol) in dimethylformamide (50 μL) was mixed with iso-1 cytochrome *c* (1.3 mg, 1.0 × 10^{-4} mmol) in 20 mM sodium dihydrogen phosphate, 20 mM ethylenediaminetetraacetic acid, 5% dimethylformamide, pH 7.0 (1 mL). The mixture was stirred at room temperature for 21.5 h and concentrated. The conjugate was then purified in 20 mM sodium dihydrogen phosphate buffer, pH 7.0 using size-exclusion chromatography (SEC, Sephadex G-25 superfine, 5 mL, GE Healthcare), concentrated and dialysed extensively into water (molecular weight cut-off 3 kDa) yielding conjugate (1.3 × 10^{-5} mmol, 13%). MS (MALDI) *m/z* 13,173 ($[M + H]^+$ requires 13,169).

Determination of encapsulation efficiency (EE%).

With the following representative calculation, the encapsulation efficiency (EE%) was determined for dye labelled cytochrome *c* and amGFP.

EE% = total number of enzymes encapsulated/number of enzymes added

For 5 μM of cyt *c* added for a 200 μL buffer solution:

$n_{cyt\ c\ added}$ = 200*10^{-6} L × 5*10^{-6} M = 1*10^{-9} mol

Now, enzymes encapsulated determined by estimating total fluorescence of sample after dialysis; for 1 a.u. fluorescence emission intensity (λ_{ex} = 488 nm, λ_{em}= 513 nm), equivalent to 4.2*10^{-3} μM cyt *c*-dye (determined experimentally). Therefore, since sample has fluorescence intensity of 849 a.u. in PBS (166μL) after dialysis,

$n_{encapsulated}$ = 849*4.2*10^{-9} μM*166*10^{-6} L = 5.9*10^{-10} mol

EE% = ($n_{encapsulated}$/$n_{cyt\ c\ added}$)*100 = (5.9*10^{-10}/1*10^{-9})*100 = 59%

Calculation of positive charge composition, F^+.

The composition of charge ratio is defined as eq. 1.[6]

$$F^+ = 1 - F^- = \frac{[+\text{charges}]}{[+\text{charges}] + [-\text{charges}]} \qquad (1)$$

At pH 7.2, it was assumed that the poly(acrylic acid) block was 70% partially ionised.[7] Additionally, yeast iso-1 cytochrome *c* had +14 charges (14 lysine residues)[8] and amGFP had +17 charges (17 lysine residues).[9]

Measurement of activity by ABTS assay[10]

The catalytic activity of yeast iso-1 cytochrome *c* was measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). A stock solution of 100 mM hydrogen peroxide was prepared by dilution of 9.4 μL of 30% hydrogen peroxide to 1 mL with ultra pure water. A second stock solution of 20 mM ABTS was prepared by dissolution of 6.9 mg ABTS in 629 μL of 20 mM sodium dihydrogen phosphate, pH 7.0. Activity of solution (bulk) cytochrome *c* and encapsulated cytochrome *c* (PS_{140}-*b*-PAA_{48}) were measured at target concentrations of 200 μM ABTS, 0.5 μM cytochrome *c*/encapsulated cytochrome *c* and 1 mM hydrogen peroxide by adding 1 μL of 20 mM ABTS, 54 μL of 0.93 μM cytochrome *c*/encapsulated cytochrome *c* to 44 μL of 20 mM sodium dihydrogen phosphate, pH 7.0 and initiating the reaction with 1 μL 1 mM hydrogen peroxide. The reactions were monitored by increasing UV absorbance at 415 nm.

Optimisation of polymersome formation

In order to determine optimum conditions for polymersome formation, such as minimal structural defects and larger encapsulation volumes in a sample population, different salt buffer conditions and temperatures were selected (Table S1). It can be seen that the optimum parameters required for polymersome preparation was room temperature incubation in the presence of phosphate buffered saline (PBS). It is interesting to observe that higher incubation temperatures and lower salt concentration results in poorly formed polymersomes with smaller vesicle diameters. Lower salt concentration buffers (20 mM NaH_2PO_4, pH 7) reduces the degree of electrostatic repulsion causing smaller vesicles to form compared to PBS (pH 7.2) with sizes 162±61 nm and 376±259 nm at room temperature, respectively. In addition, the increase in buffer temperature increases the rate of evaporation of the organic

co-solvent plasticiser (THF). As a result, the vesicles are 'frozen' over a shorter time scale compared to ambient temperature due to decreasing core-chain mobilities leading to smaller vesicles being formed.[11]

Table S1 Summary of salt buffer and temperature effects on polymersome formation.

Enzyme [a]	Buffer	T/°C	Size/nm	EE%[b]
cyt *c*	20 mM NaH_2PO_4, pH 7	25	162±61	55±1
cyt *c*	PBS, pH 7.2	25	376±259	66±7
cyt *c*	20 mM NaH_2PO_4, pH 7	40	96±25	-
cyt *c*	PBS, pH 7.2	40	267±193	-
amGFP	PBS, pH 7.2	25	308±136	35±1

[a] Enzymes prepared at 5 μM concentration. [b] Determined for optimally formed polymersomes. EE% estimated using fluorescently labelled cyt *c*.

References

1. J. R. Peterson and P. Thordarson, *Chiang Mai J. Sci.*, 2009, **26**, 236-246.
2. C. V. Iancu, W. F. Tivol, J. B. Schooler, D. P. Dias, G. P. Henderson, G. E. Murphy, E. R. Wright, Z. Li, Z. Yu, A. Briegel, L. Gan, Y. He and G. J. Jensen, *Nat. Protoc.*, 2006, **1**, 2813-2819.
3. P. M. Frederik and D. H. W. Hubert, *Methods Enzymol.*, 2005, **391**, 431-448.
4. C. D'Angelo, A. Denzel, V. Vogt, M. V. Matz, F. Oswald, A. Salih, G. U. Nienhaus and J. Wiedenmann, *Mar. Ecol. Prog. Ser.*, 2008, **364**, 97-106.
5. J. Wiedenmann, A. Schenk, C. Röcker, A. Girod, K. D. Spindler and G. U Nienhaus, *Proc. Natl. Acad. Soc. U. S. A.*, 2002, **99**, 11646-11651.
6. S. Linhoud, R. de Vries, W. Norde and M. A. C. Stuart, *Biomacromolecules*, 2007, **8**, 2219-2227.
7. J. Choi and M. F. Rubner, *Macromolecules*, 2005, **38**, 116-124.
8. G. V. Louie and G. D. Brayer, *J. Mol. Biol.*, 1990, **214**, 527-555.
9. C. D'Angelo, A. Denzel, V. Vogt, M. V. Matz, F. Oswald, A. Salih, G. U. Nienhaus and J. Wiedenmann, *Mar. Ecol. Prog. Ser.*, 2008, **364**, 97-106.
10. J. R. Peterson, T. A. Smith and P. Thordarson, *Chem. Commun.*, 2007, 1899-1901.
11. L. Zhang and A. Eisenberg, *J. Am. Chem. Soc.*, 1996, **118**, 3168-3181.

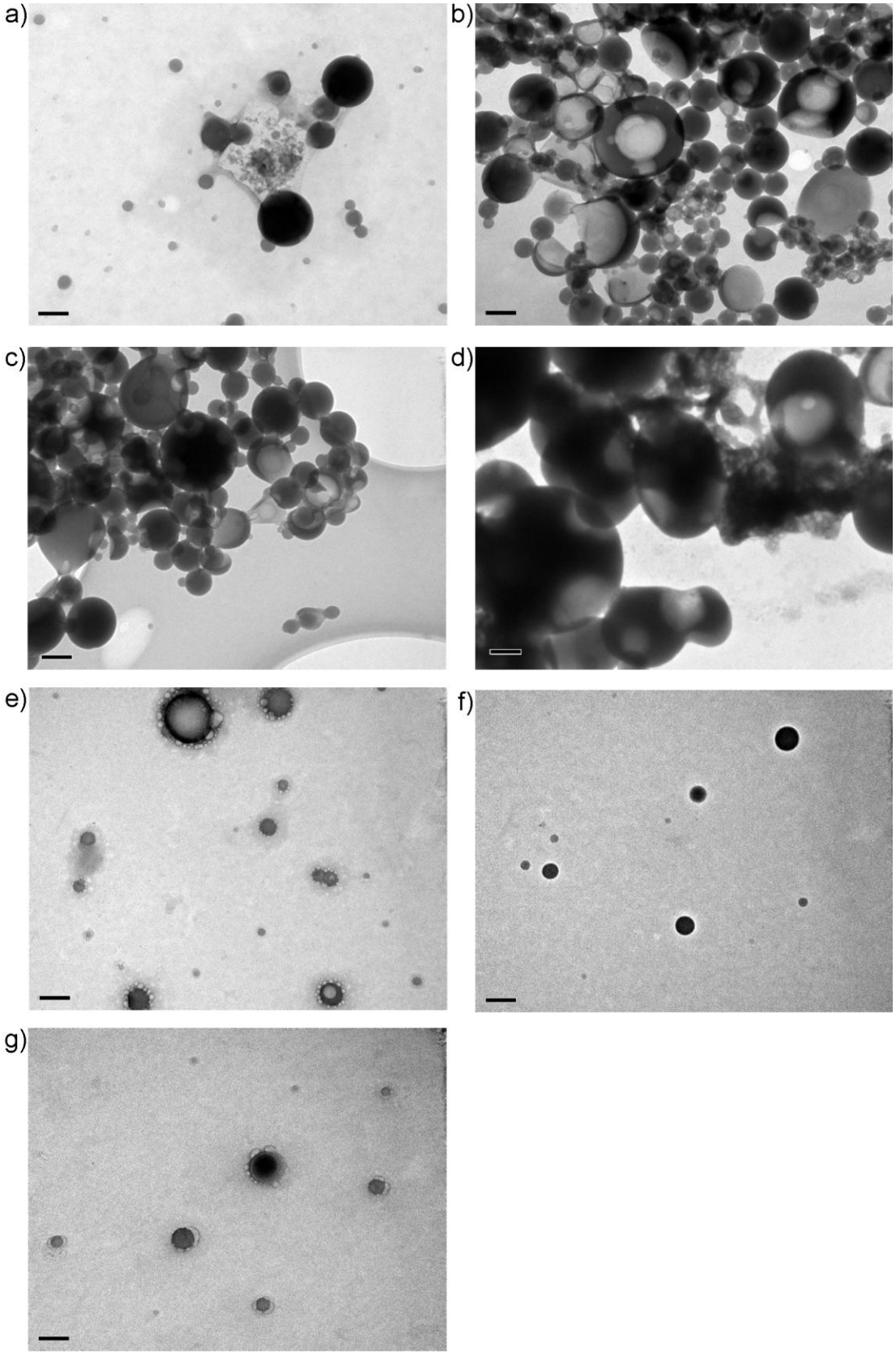


Figure S1. *TEM micrographs of diblock copolymer aggregates in the presence of 5 μM additives in PBS (25 °C). a) PBS control (no enzyme), b) cyt* c*, c) amGFP, d) poly-L-lysine, e) Mb, f) BSA, g) calmodulin. Scale bars: 200 nm.*

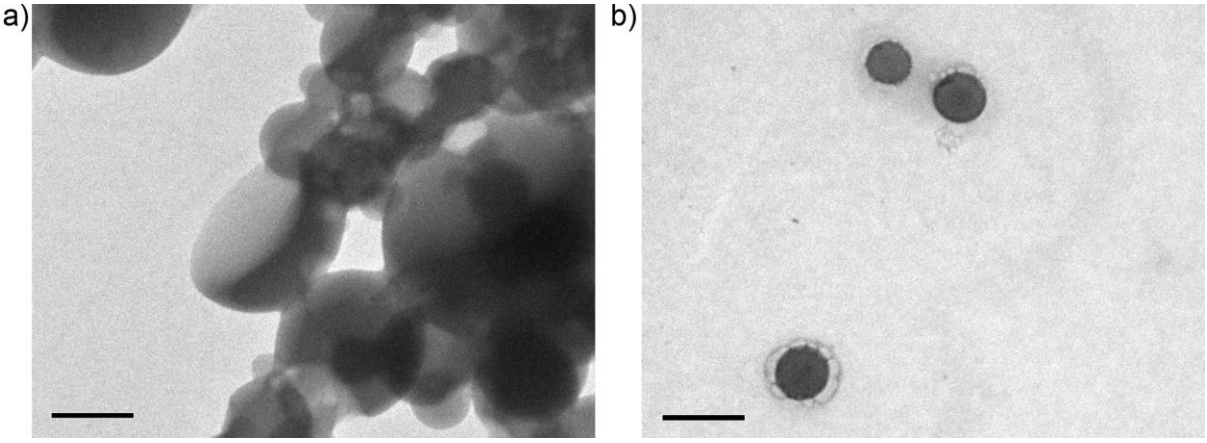


Figure S2. *TEM micrographs of diblock copolymer aggregates in the presence of 5 μM cyt* c *after post-addition to polymer micelle mixture in PBS (25 °C). a) 30 min. b) 24 h. Scale bars: 200 nm.*

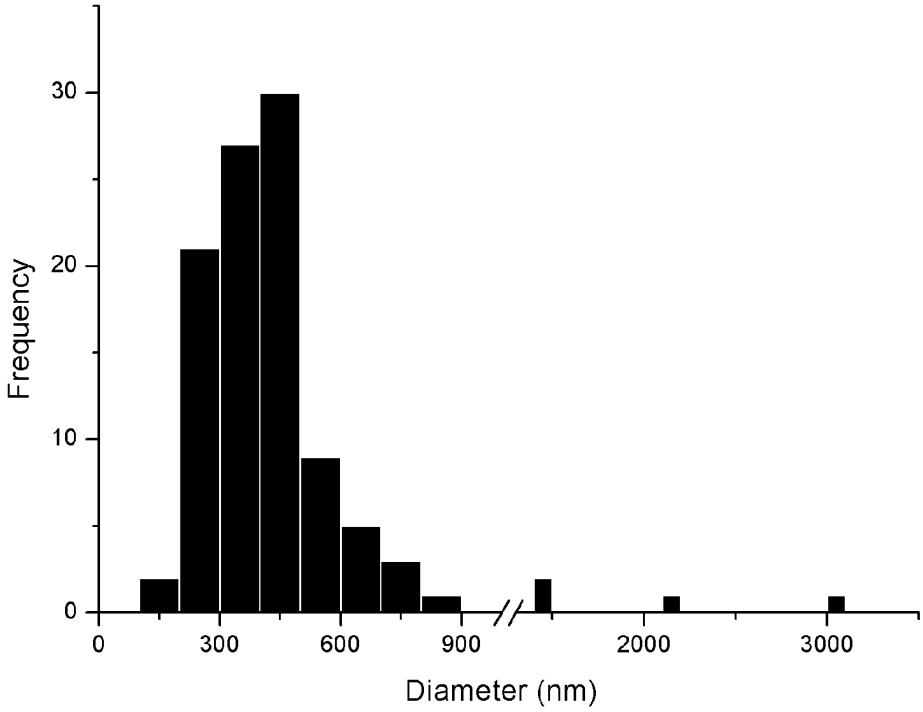


Figure S3. *Representative histogram of PS_{140}-b-PAA_{48} polymersomes for 5 μM cyt c in PBS at 25 °C.*

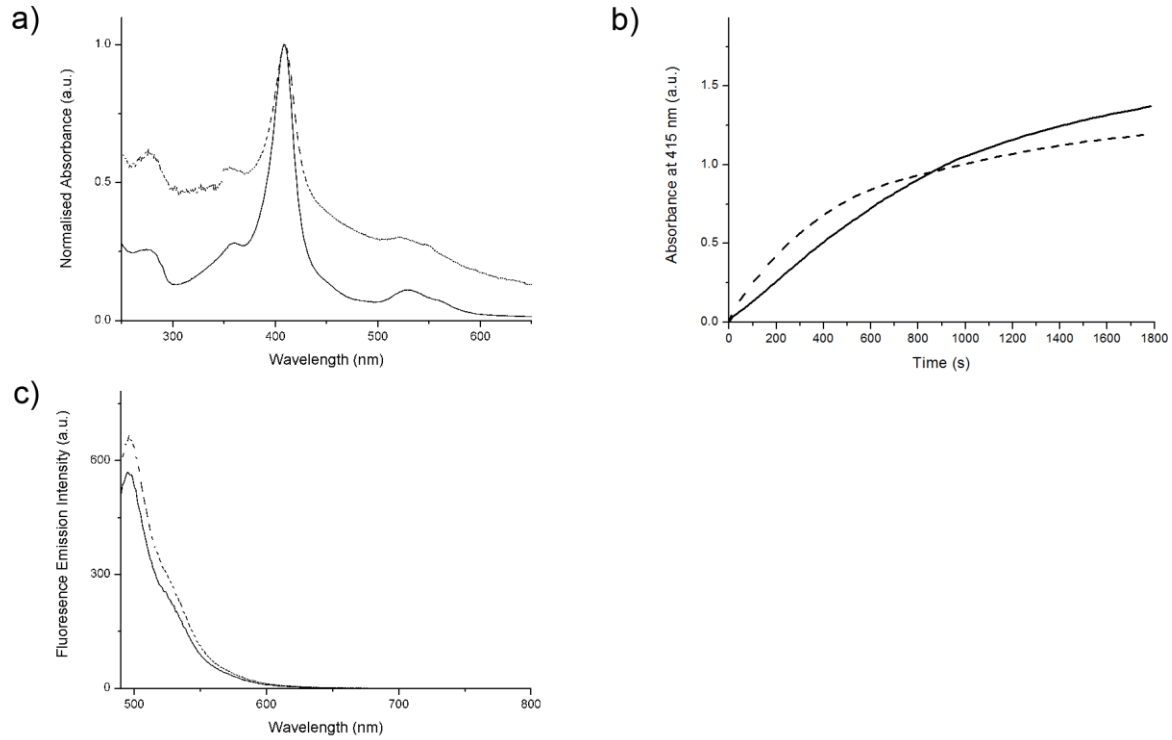


Figure S4. *a) UV-Vis spectra of soret band (410 nm) of cytochrome* c. *Solution cyt* c *(solid line), encapsulated cyt* c *(dashed line). Sloping baseline for encapsulated cyt* c *due to scattering by polymersome aggregates. b) Catalytic activity of solution cyt c (solid line) and encapsulated cyt c (dashed line) by monitoring oxidation of ABTS at 415 nm. c) Fluorescence emission profiles of amGFP excited at 476 nm with emission maxima 497 nm. Solution amGFP (solid line), encapsulated amGFP (dashed line).*