Supplementary Information for "Permeable Protein-Loaded Polymersome Cascade Nanoreactors by Polymerization-Induced Self-Assembly"

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Experimental Section

Methods and materials

Materials

The monomer 2-hydroxypropyl methacrylate (mixture of isomers, HPMA) was purchased from Alfa Aesar and was passed through a column of basic alumina prior to use. The synthesis of the PEG_{113} mCTA from PEG_{113} monomethyl ether and 4-cyano-4-(((ethylthio)carbonothioyl)thio)pentanoic acid (CEPA) has been described in a previous report.[1] The reagents 3,3'-dimethoxybenzidine (DMB) and D-glucose were purchased from Sigma Aldrich and used as received. Hydrogen peroxide (35% v/v) was purchased from Lancaster Synthesis and used as received. The enzymes peroxidase from *Amoracia rusticana* (type VI, essentially salt free) (HRP) and glucose oxidase from *Aspergillus niger* (type VII) (GOx) were purchased from Sigma Aldrich, divided into aliquots at 200 U mL^{-1} in deionized water and stored at -20 °C. Aliquots were thawed at room temperature prior to use on the day of thawing. Formvar and lacey-carbon coated copper grids were purchased from EM Resolutions.

The pWALDO plasmid encoding for a hexahistidine-tagged enhanced GFP was kindly donated by Dr. Elizabeth Fullam, University of Warwick, UK. Monoclonal Anti-polyHistidine antibody produced in mouse, clone HIS-1, ascites fluid and goat anti-Mouse IgG (H+L) secondary antibody (AP-conjugated) were purchased from Sigma Aldrich and used as received. The antibodies goat anti-rabbit IgG H+L (HRP conjugated), goat anti-horseradish peroxidase, rabbit anti-glucose oxidase and donkey anti-goat IgG H+L (HRP conjugated) were purchased from Abcam and used as received. *Escherichia coli* (*E. coli*) BL21(DE3) cells were purchased from New England Biolabs.

Characterization techniques

SEC analysis of the diblock copolymers was performed on a Varian PL-GPC 50 Plus instrument fitted with mixed C columns and RI and UV detectors using 5 mM NH_4BF_4 in DMF as the eluent. Molecular weight distributions were calculated using poly(methyl methacrylate) standards.

DLS analysis of the vesicles was performed on a Malvern Zetasizer Nano S instrument. Purified samples were diluted with filtered deionized water (0.45 μm, nylon) and the diluted samples were not filtered prior to analysis so as to ensure larger structures remained in solution. Average z-average hydrodynamic radii (D_H) were calculated from 5 repeat measurements.

Dry state stained TEM analysis was performed on either a JEOL 2100 FX or a JEOL 2000 FX microscope. Purified samples were diluted with deionized water then deposited onto formvar grids. After roughly 1 min, excess sample was blotted from the grid and the grid stained with an aqueous 1 wt% uranyl acetate solution for 1 min prior to blotting, drying and microscopic analysis. Purified samples for cryo-TEM analysis were prepared at 5.5 mg mL^{-1} in 100 mM pH 5.5 PB by depositing 8 µL sample onto a lacey-carbon grid followed by blotting for approximately 4 s and plunging into a pool of liquid ethane cooled using liquid nitrogen to vitrify the sample. Transfer into a pre-cooled cryo-TEM holder was performed under liquid nitrogen temperatures prior to microscopic analysis under liquid nitrogen temperatures.

Kinetic colorimetric analyses were performed in 96-well Nunc plates and measured on a BMG Labtech FLUOstar OPTIMA plate reader running in absorbance mode with a filter of 492 nm. Absorbance values at this wavelength were measured every minute. Measurements were performed in at least triplicate.

Fluorescence spectroscopy was performed on an Agilent Cary Eclipse fluorescence spectrophotometer set to the medium voltage preset and with slit widths of 5 nm. Preparation of the fluorescence microscope slides was performed by cleaning with a lint-free cloth and plasma cleaning the slide for 1 min. The sample (10 µL) was deposited into the center of the slide and dried under a gentle flow of compressed air to fix the vesicles to the slide. Images were obtained using a cooled pE300 camera coupled with an Olympus CKX41 microscope at a magnification of 40x using FITC channel (Ex./Em. = 490/525 nm).

Recombinant GFP expression and purification

A pWALDO plasmid encoding for a hexahistidine-tagged GFP was transformed into competent *Escherichia coli* (*E. coli*) BL21(DE3) cells. A single colony was selected and grown overnight in 50 mL of Lysogeny broth (LB)-medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin under continuous shaking (37 °C, 180 prm). 5 mL of the preculture was then added to 500 mL of LB-medium in a 2 L Erlenmeyer flask and grown for 4 hours under continuous shaking (37 °C, 180 rpm). The temperature was reduced to 16 °C and cells were incubated for an hour further. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the cells to a final concentration of 1 mM. The protein overexpressed overnight, following which the cells were separated from the medium by centrifugation (4 °C, 4000 g, 30 minutes).

Pelleted cells were resuspended in 15 mL phosphate buffered saline (PBS) (18.2 MΩ·cm mean resistivity, [NaCl] = 0.138 M, [KCl] = 0.0027 M, and pH 7.4). Pierce protease inhibitor mini-tablets were added to the suspension and it was passed through a STANSTED 'Pressure Cell' FGP12800 homogeniser to undergo lysis. The cell lysate was centrifuged (4 °C, 14,000 g, 30 minutes) and the supernatant filtered through a syringe filter (0.2 µm) and passed through a pre-equilibrated (20 mL PBS) IMAC Sepharose 6 Fast Flow (GE Healthcare) column charged with Ni(II) ions. The column was washed first with 10 column volumes of 20 mM imidazole in PBS, then with 5 column volumes of 50 mM imidazole in PBS. 250 mM and 1000 mM imidazole in PBS were used to elute bound GFP and the imidazole removed from the fractions using PD10 desalting columns (GE Healthcare). Samples for western blot analysis were resolved on a polyacrylamide gel, transferred to a membrane and detected a using primary (monoclonal anti-polyhistidine) antibody and a secondary (goat anti-mouse IgG (H+L)) antibody. The protein concentration determined using Thermo Scientific Pierce BCA assay kit according to the supplier's instructions. The GFP containing PBS solution was aliquoted into 1.5 mL microcentrifuge tubes (200 µL), snap-frozen in liquid nitrogen and stored at -80 °C.

Particle synthesis

Preparation of protein-loaded vesicles. A typical synthesis for the preparation of HRP-loaded vesicles was as follows.[2] PEG_{113} mCTA (9.1 mg) was dissolved in deionized water (800 µL) and HPMA (100 mg) added. Once homogeneous, 100 µL of a 200 U mL^{-1} solution of HRP in deionized water was added and the solution was sealed in a scintillation vial with a stirrer bar. After degassing the solution by purging with nitrogen for 10 min, the sealed vial was stirred at 37 °C and irradiated with a 400-410 nm light source for 70 min. The opaque vesicle solution was diluted by a factor of 10 with 100 mM pH 5.5 PB and purified by centrifugation (16,000 x g for 10 min) followed by resuspension in PB and further purified by preparative SEC (superdex increase 200, 100 mM pH 5.5 PB). The combined vesicle fractions were then diluted to 5.5 mg mL^{-1} (20 times dilution from the original reaction mixture) prior to the colorimetric assay. GOx-loaded vesicles were prepared in an identical manner. In the case of the desalted purified GOx, the enzyme solution was purified by dialysis through a spin filter (MWCO = 5 kDa) prior to use. For GFP-loaded vesicles, a final feed concentration of 0.315 mg mL^{-1} protein was used and the particles were purified from the free protein by 3 centrifugation/resuspension cycles in 100 mM pH 5.5 PB.

Kinetic colorimetric analyses

Activity of HRP-loaded vesicles. The purified sample at 20x dilution from the initial PISA synthesis in 100 mM pH 5.5 PB (120 µL) was diluted with 100 mM pH 5.5 PB (20 µL) in a 96-well plate well. 2 mM DMB (40 µL) was added. Finally, a 35% w/w aqueous solution of hydrogen peroxide (20 µL) was added and the change in absorbance at 492 nm was recorded every minute using a plate reader.

Activity of GOx-loaded vesicles. The purified sample at 20x dilution from the initial PISA synthesis in 100 mM pH 5.5 PB (120 µL) was added to a 96-well plate well. A 200 U mL^{-1} aqueous solution of fresh HRP (20 µL) and 2 mM DMB (40 µL) were added. Finally, a 30 mg mL^{-1} aqueous solution of D-glucose (20 µL) was added and the change in absorbance at 492 nm was recorded every minute using a plate reader.

Activity of the HRP-loaded vesicle and GOx-loaded vesicle cascade. A typical experiment at a 50:50 HRP:GOx ratio was as follows. Purified samples of both HRP-loaded vesicles (70 µL) and GOx-loaded vesicles (70 µL), each at 20x dilution from the initial PISA synthesis in 100 mM pH 5.5 PB, were added to a 96-well plate well. 2 mM DMB (40 µL) was added. Finally, a 30 mg mL^{-1} aqueous solution of D-glucose (20 µL) was added and the change in absorbance at 492 nm was recorded every minute using a plate reader.

Calculation of loading efficiencies

Western blot analysis of the enzyme-loaded vesicles. In a typical experiment using HRP-loaded vesicles, the sample was mixed in a 1:1 ratio with SDS loading buffer, followed by heating and shaking for 15 min at 80 °C using a thermomixer. The sample was ran on a 4-20% poly(acrylamide) gel at 200 V and 400 mA for 30 min using tris glycine SDS as the running buffer. The gel was transferred to a nitrocellulose membrane using 20% methanol tris glycine as the transfer buffer at 100 V and 400 mA for 45 min. The membrane was washed with tris buffered saline (TBS)/tween and incubated for 1 h with milk/TBS/tween. The membrane was then incubated with goat anti-horseradish peroxidase in milk/TBS/tween at an antibody dilution of 1:1,000 overnight. After washing 3 times with TBS/tween, the membrane was incubated with donkey anti-goat IgG H+L (HRP conjugated) in milk/TBS/tween at an antibody dilution of 1:1,000 for 1 h. After washing 3 times with TBS/tween, the membrane was incubated with a luminol/peroxide chemiluminescent reagent for 2 minutes, followed by digital visualization using a 30 s exposure time. For GOx, rabbit anti-glucose oxidase and goat anti-

rabbit IgG H+L (HRP conjugated) were used as the primary and secondary antibodies respectively.

Western blot data processing. The pixel area of each band was determined using ImageJ software and a calibration curve for each enzyme was determined using the dilution series of known concentration. This allowed for the determination of the sample protein concentration using the measured pixel area of the sample protein band.

Supplementary SEC data

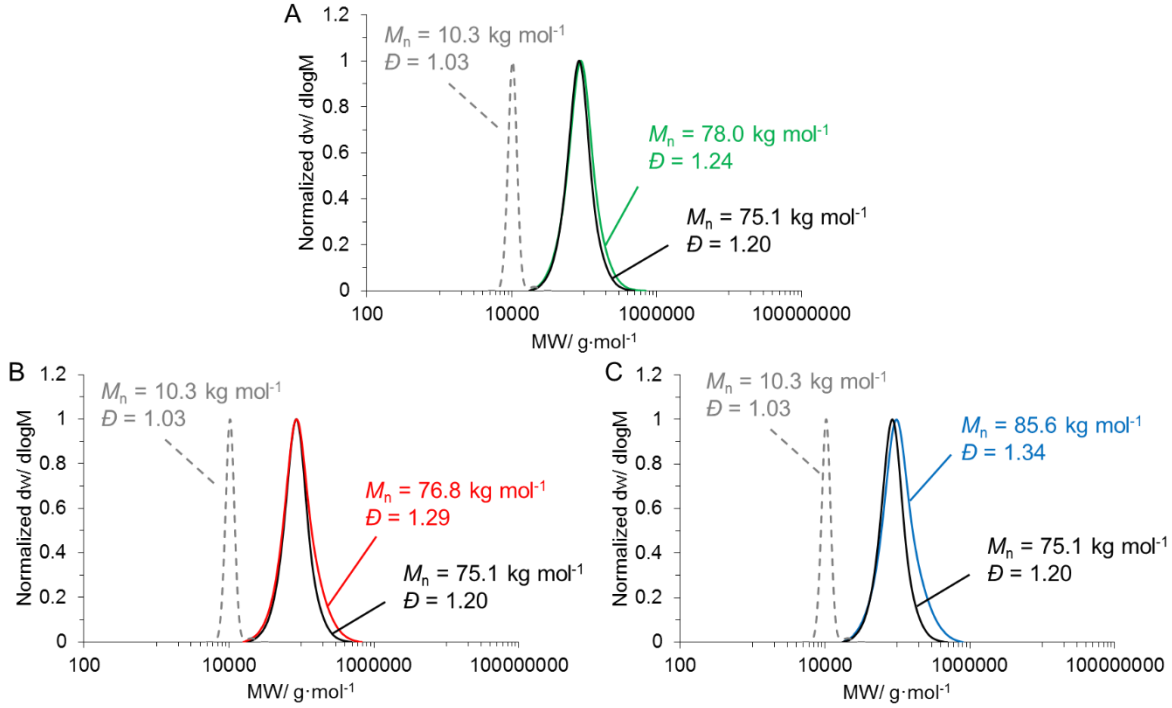

Figure S1. SEC RI traces of the PEG_{113} mCTA (gray dashed line, all panels) and the PEG_{113}-*b*-$PHPMA_{400}$ diblock copolymers (solid lines, all panels) comprising the protein-loaded vesicles used in this study. Empty vesicles (black trace, all panels), GFP-loaded vesicles (green trace, panel A), HRP-loaded vesicles (red trace, panel B) and GOx-loaded vesicles (blue trace, panel C) are shown, along with their calculated M_n and $Đ$ values. 5 mM NH_4BF_4 in DMF was used as the eluent and molecular weight distributions were calculated from poly(methyl methacrylate) standards.

Supplementary data for empty vesicles

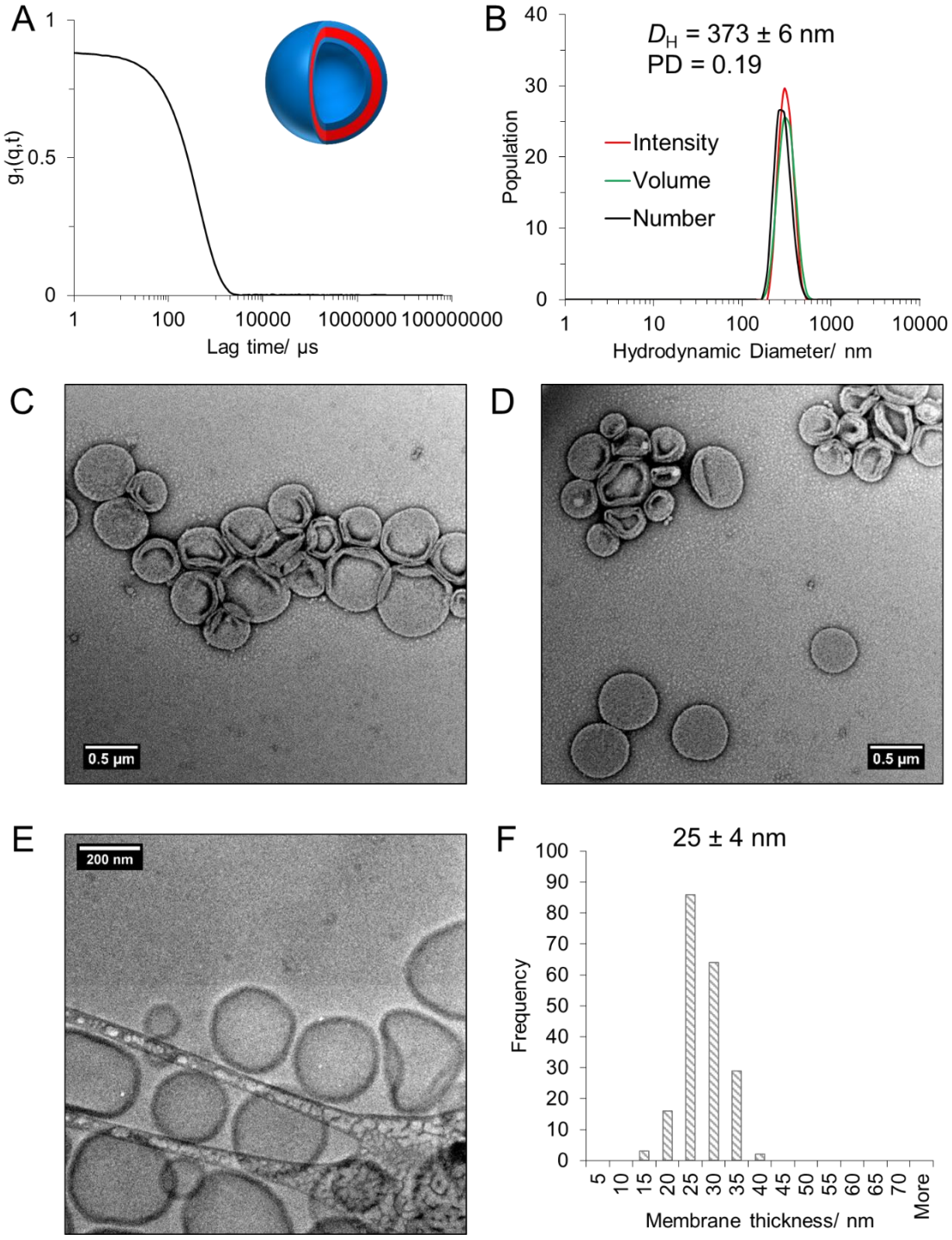

Figure S2. Characterization of empty vesicles. DLS autocorrelation function (A) and distribution (B) of empty vesicles in pH 5.5 phosphate buffer. The D_H and PD are shown. The error represents the standard deviation from 5 repeat measurements. Representative dry state TEM images of samples stained with 1 wt% uranyl acetate (C and D). Representative cryo-TEM image of empty vesicles in pH 5.5 phosphate buffer (E) and distribution of membrane thicknesses measured from statistical analysis (F). The error shows the standard deviation from 200 particle membranes.

Supplementary data for GFP-loaded vesicles

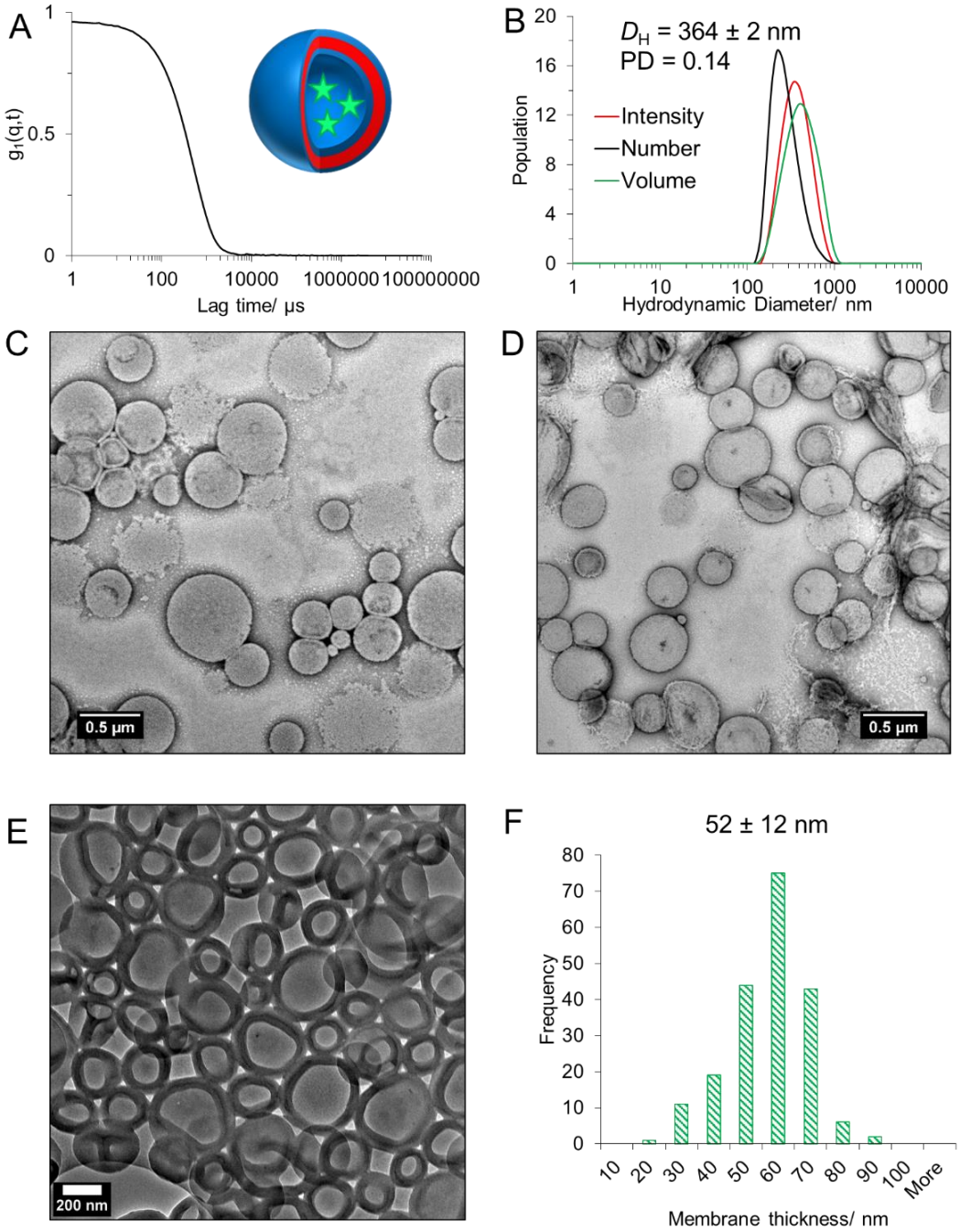

Figure S3. Characterization of GFP-loaded vesicles after purification. DLS autocorrelation function (A) and distribution (B) of GFP-loaded vesicles in pH 5.5 phosphate buffer. The D_H and PD are shown. The error represents the standard deviation from 5 repeat measurements. Representative dry state TEM images of samples stained with 1 wt% uranyl acetate (C and D). Representative cryo-TEM image of GFP-loaded vesicles in pH 5.5 phosphate buffer (E) and distribution of membrane thicknesses measured from statistical analysis (F). The error shows the standard deviation from 200 particle membranes.

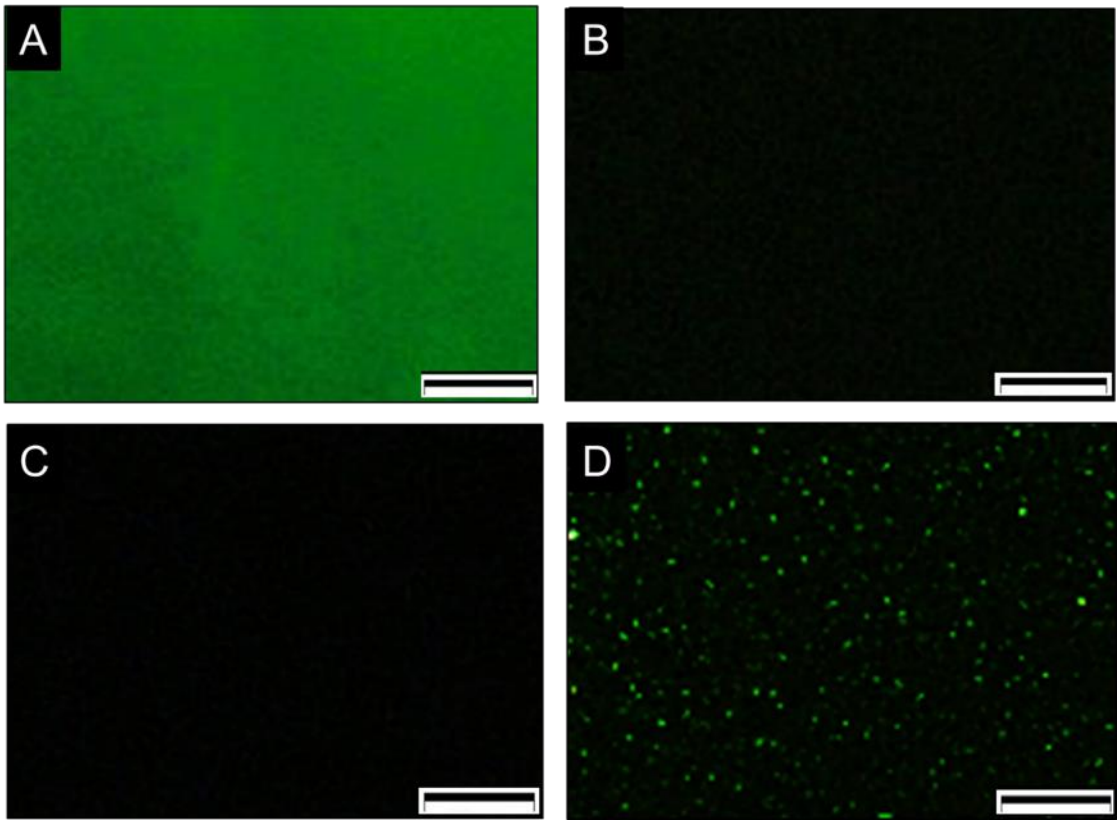

Figure S4. Representative fluorescence microscopy images of free GFP (A), blank vesicles containing no encapsulated protein (B) and blank vesicles purified from 2 U mL^{-1} GFP after 70 min light irradiation at 37 °C (C). The GFP-loaded vesicle sample is shown in panel D for comparison. The scale bar in each case is 5 μm.

Supplementary data for HRP-loaded vesicles

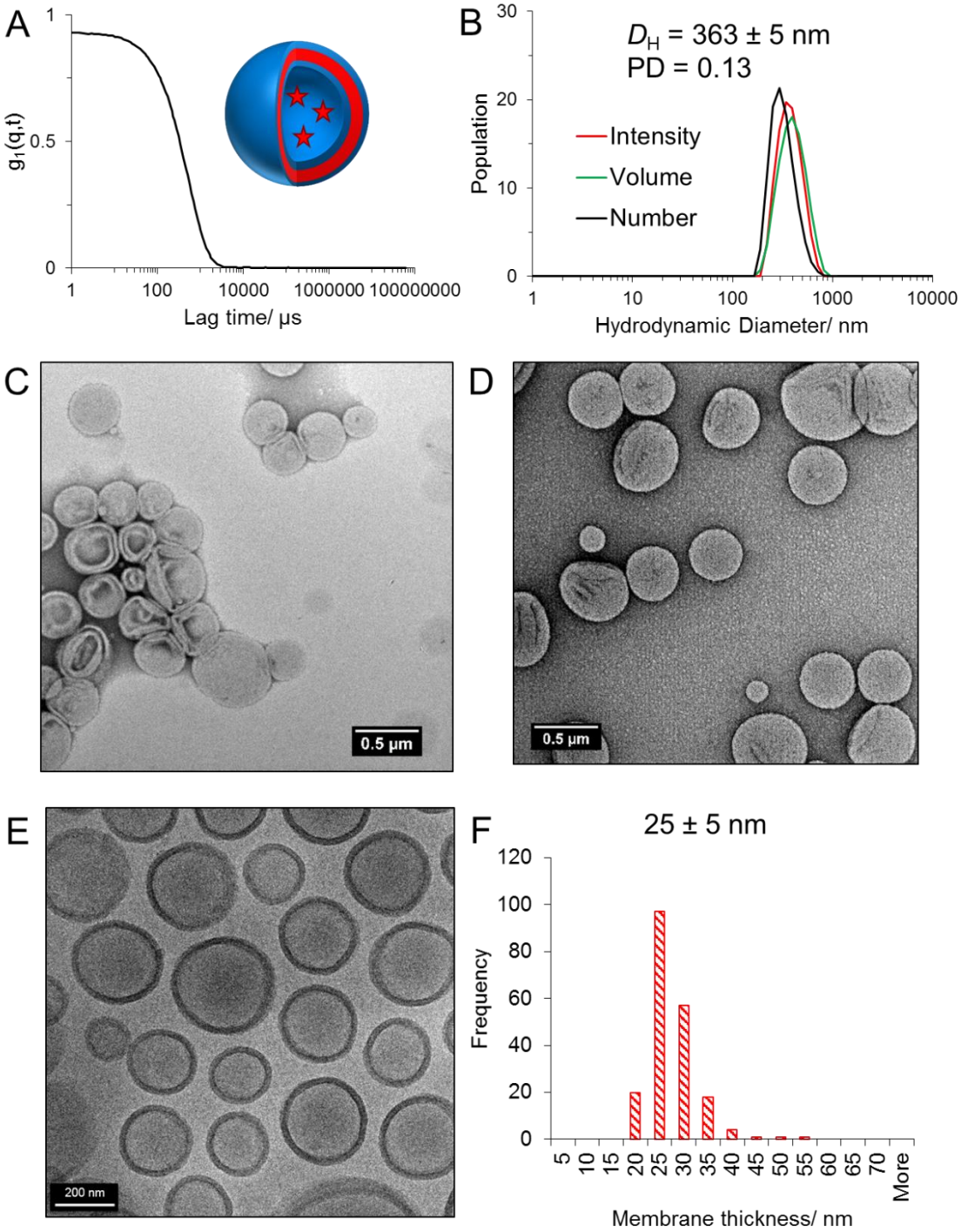

Figure S5. Characterization of HRP-loaded vesicles after purification. DLS autocorrelation function (A) and distribution (B) of HRP-loaded vesicles in pH 5.5 phosphate buffer. The D_H and PD are shown. The error represents the standard deviation from 5 repeat measurements. Representative dry state TEM images of samples stained with 1 wt% uranyl acetate (C and D). Representative cryo-TEM image of HRP-loaded vesicles in pH 5.5 phosphate buffer (E) and distribution of membrane thicknesses measured from statistical analysis (F). The error shows the standard deviation from 200 particle membranes.

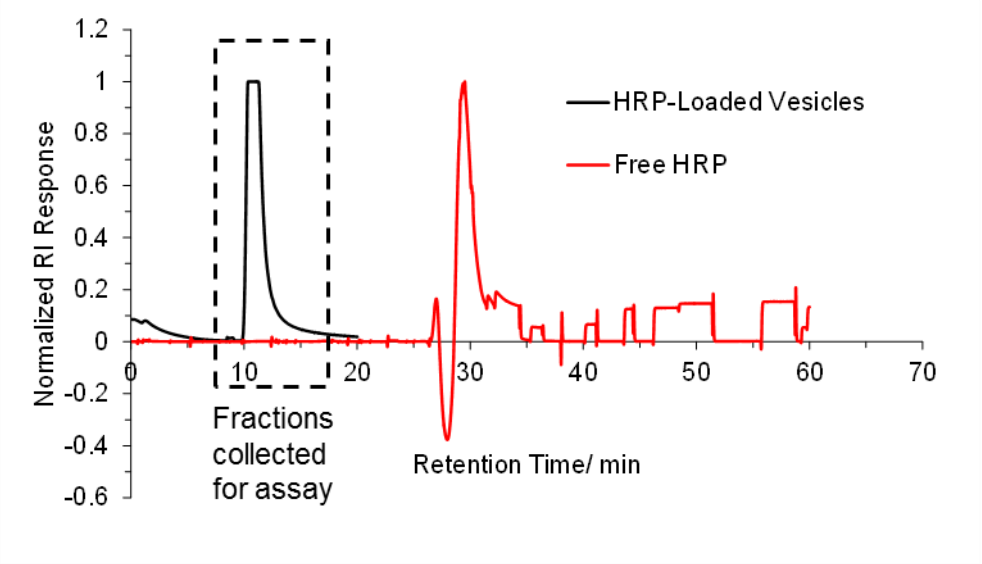

Figure S6. Normalized preparative SEC RI traces of HRP-loaded vesicles (black trace) and that of the free HRP enzyme (red trace) in 100 mM pH 5.5 phosphate buffer. In each case, the injection volume was 1 mL.

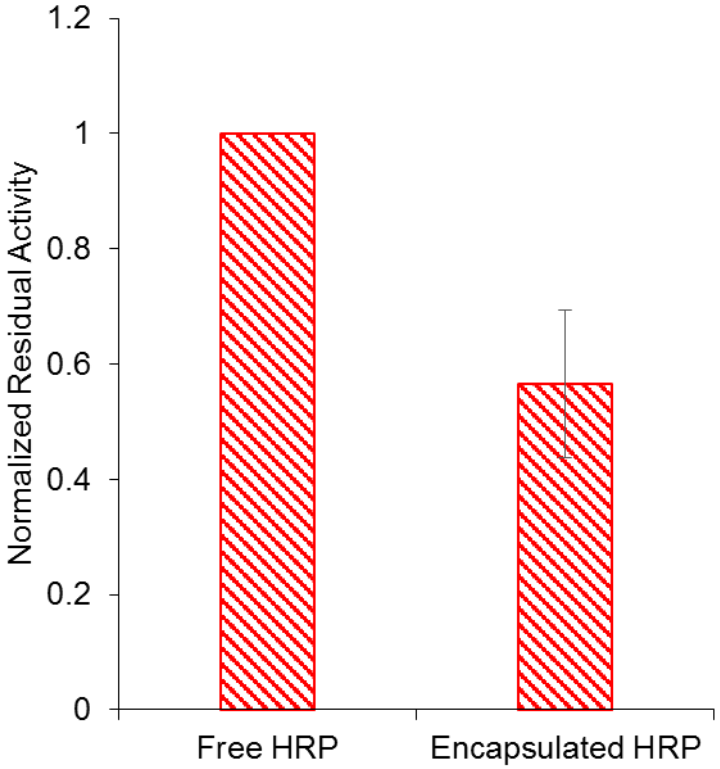

Figure S7. Residual activity of HRP-loaded vesicles based on the maximum reaction velocity relative to that of the free enzyme. The error represents the standard deviation from 4 repeats.

Supplementary data for GOx-loaded vesicles and cascade activity

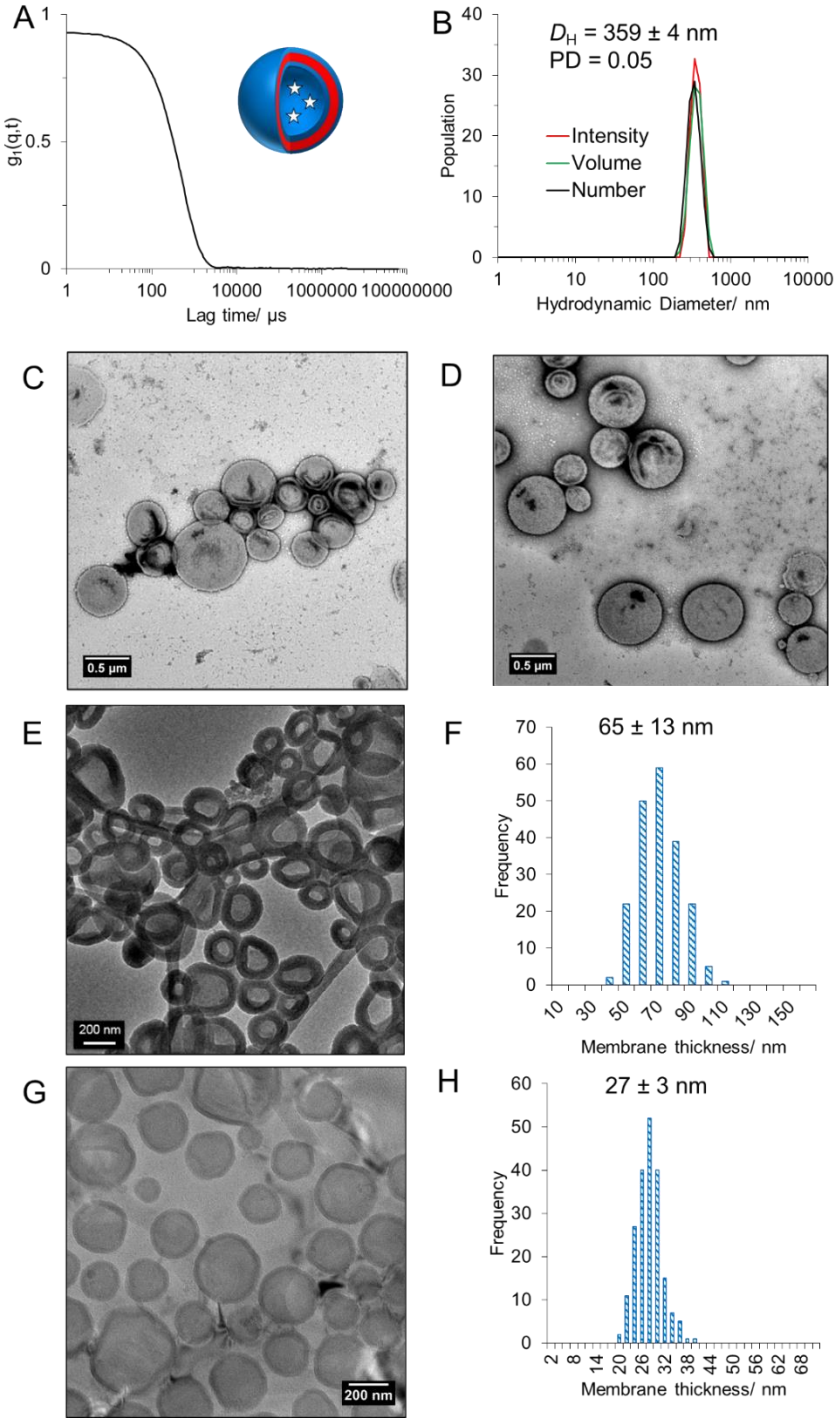

Figure S8. Characterization of GOx-loaded vesicles after centrifugation/resuspension and preparative SEC. A and B: DLS analysis of GOx-loaded vesicles in pH 5.5 phosphate buffer. The D_H and PD are shown. The error represents the standard deviation from 5 repeat measurements. C and D: Representative dry state TEM images of samples stained with 1 wt% uranyl acetate. E and F: Representative cryo-TEM image of commercial GOx-loaded vesicles in pH 5.5 phosphate buffer and distribution of membrane thicknesses measured from statistical analysis (bottom). G and H: Representative cryo-TEM image in pH 5.5 phosphate buffer of vesicles loaded with GOx previously purified by dialysis, and distribution of membrane thicknesses measured from statistical analysis. In each case, the error shows the standard deviation from 200 particle membranes.

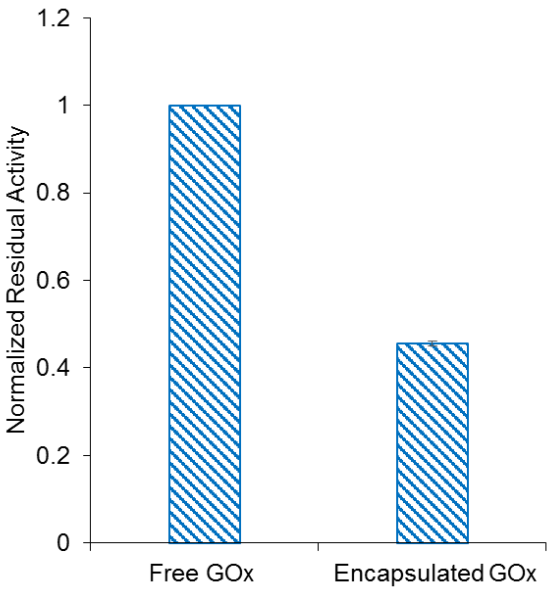

Figure S9. Residual activity of GOx-loaded vesicles based on the maximum reaction velocity relative to that of the free enzyme. The error represents the standard deviation from 4 repeats.

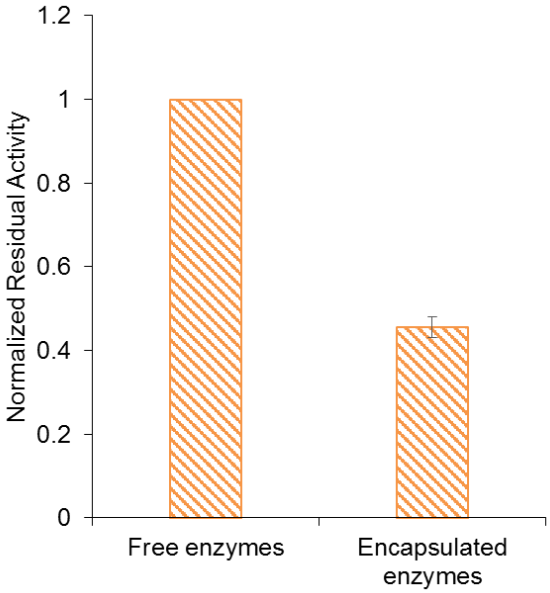

Figure S10. Residual cascade activity of enzyme-loaded vesicles based on the maximum reaction velocity relative to that of the free enzymes. The error represents the standard deviation from 4 repeats.

Calculation of loading efficiencies and comparison of activities

The concentration of the enzymes inside the vesicles was determined by Western blot analysis of the disassembled sample. The vesicles were first destroyed with surfactant to release their encapsulated enzyme before running through a poly(acrylamide) gel by electrophoresis. The gel was transferred to a nitrocellulose membrane, which was incubated with first a primary antibody specific for the protein of interest, then with a species specific secondary antibody conjugated to HRP. On incubating the prepared membrane with a chemiluminescent reagent for HRP, the blot could be developed and the bands corresponding to the protein could be visualized. For each sample, a dilution series of known concentrations was ran on the same gel, each lane corresponding to different calculated encapsulation efficiencies. By measuring the pixel area of each band using ImageJ software, a calibration curve was obtained. Figure S11 shows an example of a developed blot for the disassembled GOx-loaded vesicles along with the obtained calibration curve for the dilution series. Once the encapsulation efficiencies for these enzymes were calculated, a comparison of the activities of the encapsulated enzymes to the free enzymes could be made. The activity of an identical concentration of free enzyme was assessed and compared to the activities of the enzyme-loaded vesicles. Note that the same batch of vesicles analyzed by Western blot were used in these comparative activity assays.

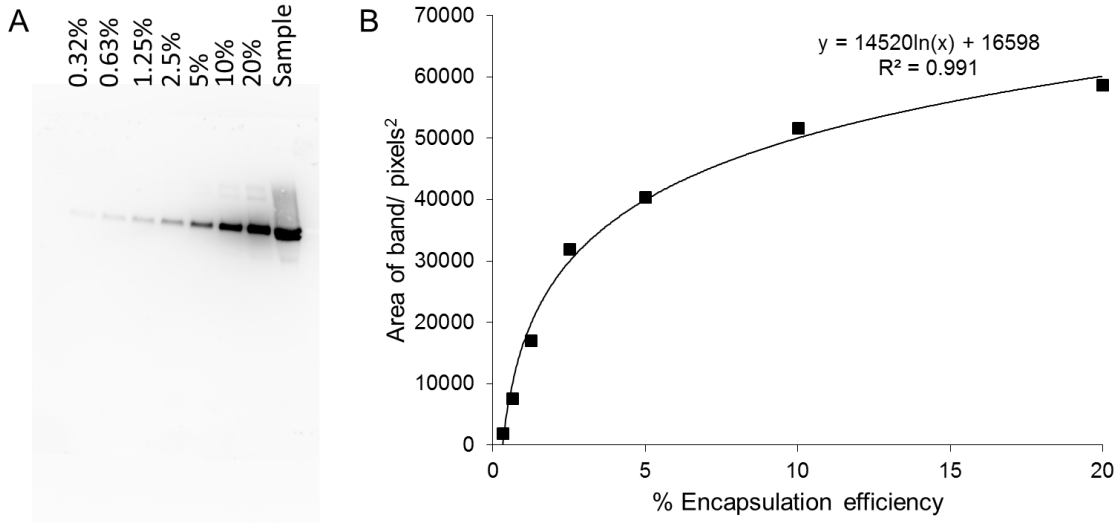

Figure S11. Western blot analysis of GOx-loaded vesicles disassembled using SDS. A: Image of the developed blot. B: Obtained calibration curve of the dilution series.

Estimation of the number of enzymes per vesicle

Assuming that the enzyme concentration ([E]) inside the vesicle and outside the vesicle were the same at the point of vesicle formation, the number of protein molecules can be roughly estimated. The number of moles (n) should be equal to the concentration of enzyme in the initial feed multiplied by the internal volume of the lumen (V_{internal}).

$$n = [\text{E}]_{\text{internal}} V_{\text{internal}} = \frac{4\pi [\text{E}]_{\text{internal}} (R_{\text{internal}})^3}{3}$$

Where

$$[\text{E}]_{\text{internal}} = [\text{E}]_{\text{external}}$$

$$R_{\text{internal}} = R_{\text{external}} - membrane\ thickness$$

R_{external} and the membrane thickness were determined by DLS and cryo-TEM, respectively.

Based on the purities and activities in $\text{U}\cdot\text{mg}^{-1}$ supplied by Sigma Aldrich, and based on an initial feed of 20 $\text{U}\cdot\text{mL}^{-1}$, the number of enzyme moles are 3.6×10^{-23} mol for HRP and 3.9×10^{-24} mol for GOx. This equates to average loadings of roughly 22 HRP proteins per vesicle and 2 GOx per vesicle.

Supplementary GFP Characterization

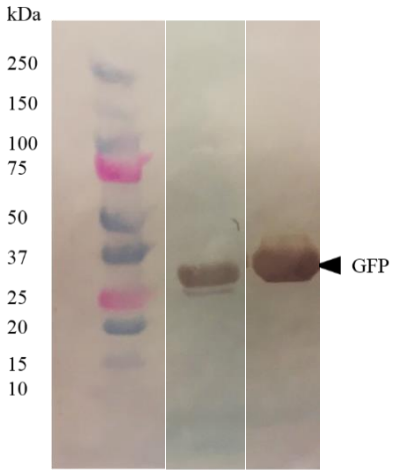

Figure S12. Western blot analysis of *E. coli* cells and GFP elutions from a Nickel-sepharose column. Purified GFP is observed in the RHS lane and non-purified GFP in the center lane. The numbers on the left represent the molecular mass in kilodaltons, relative to Precision Plus Protein^{TM} Standards (LHS lane).

References

(1) Tan, J.; Sun, H.; Yu, M.; Sumerlin, B. S.; Zhang, L. Photo-PISA: Shedding Light on Polymerization-Induced Self-Assembly. *ACS Macro Lett.* **2015**, *4*, 1249-1253.

(2) Blackman, L. D.; Doncom, K. E. B.; Gibson, M. I.; O'Reilly, R. K. Comparison of photo- and thermally initiated polymerization-induced self-assembly: a lack of end group fidelity drives the formation of higher order morphologies. *Polym. Chem.* **2017**, *8*, 2860-2871.