Electronic Supplementary Material (ESI) for ChemComm.

Microfluidic formation of proteinosomes

Martina Ugrinic[a], Adrian Zambrano[b], Simon Berger[a], Stephen Mann[c], T-Y Dora Tang[b,c*], Andrew deMello[a*]

(a) Department of Chemistry & Applied Biosciences, ETH Zurich, Vladimir Prelog Weg 1, 8093 Zurich, Switzerland; (b) Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstraße 108, 01307 Dresden, Germany; (c) Centre for Protolife Research, School of Chemistry, University of Bristol, Bristol, BS8 1TS, UK

Supplementary Figures:

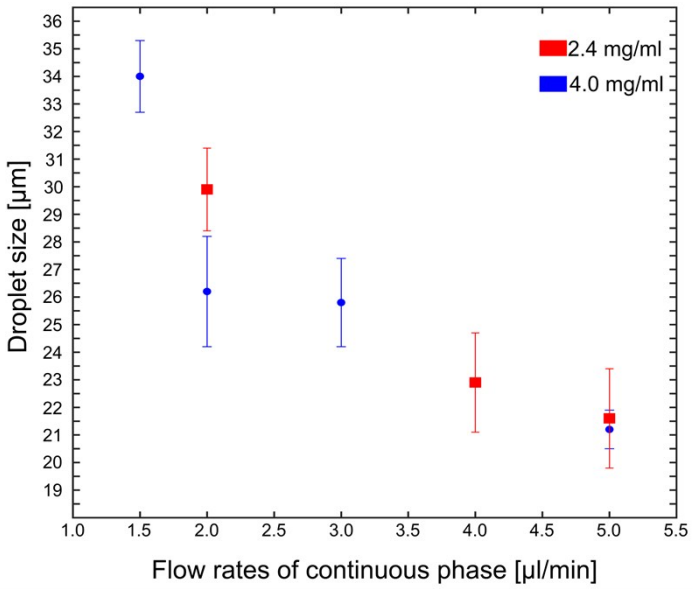

Figure S1: Controlled variation of average droplet size as a function of the volumetric flow rate of the continuous phase for GOx-NH_2/PNIPAAm water-in-oil droplets for 2.4 mg/mL (red squares) and 4 mg/mL (blue circles) of GOx-NH_2/PNIPAAm conjugate in HEPES buffer at flow rate of 1µl / min. Analysis of the data confirms that the dispersed conjugate concentration has no effect on the size of the droplet.

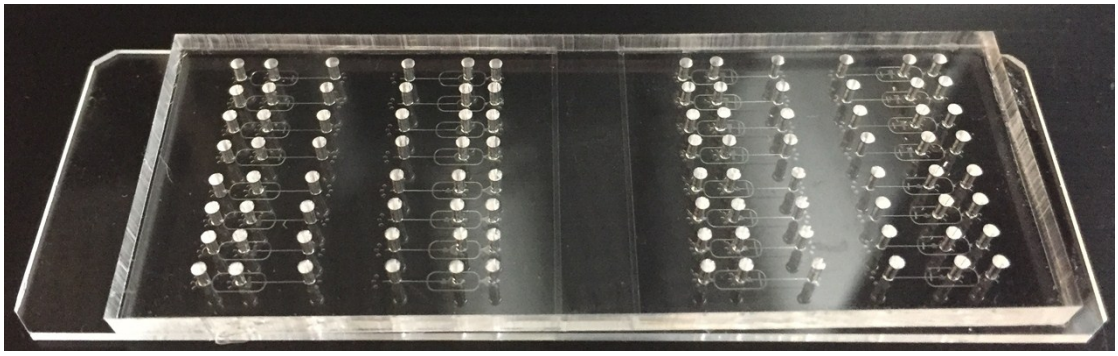

Figure S2: Assembly of 32 devices on one glass slide. Devices can be run in parallel to scale-up proteinosome production.

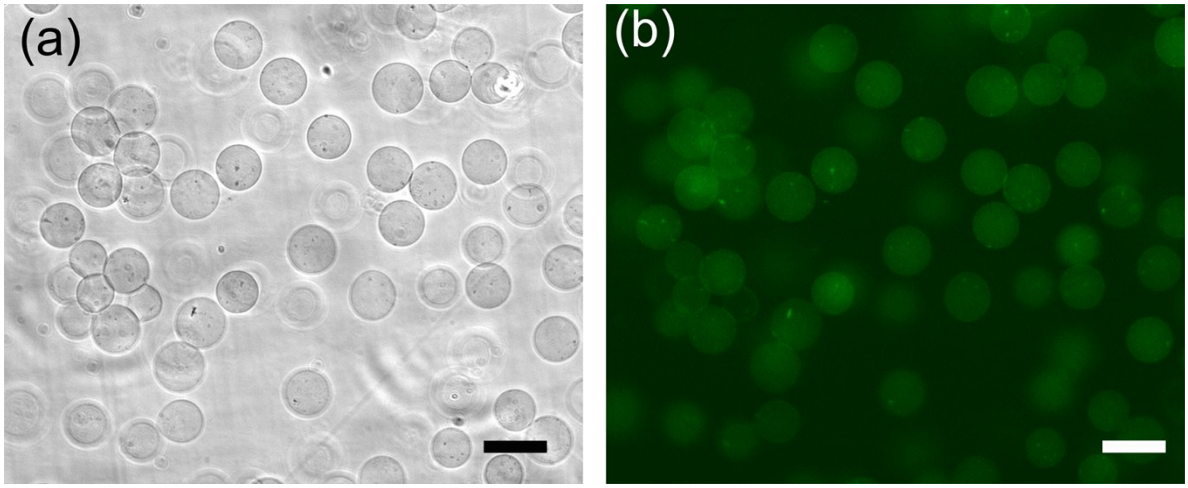

Figure S3: Phase contrast (a) and fluorescence (b) images of GOx-PNIPAAm proteinosomes with encapsulated HRP-FITC (prepared within the microfluidic device) 4 weeks after formation confirm proteinosome stability and retention of encapsulated enzymes. Proteinosomes were stored at +4°C in water. Scale bar 50 µm.

Supplementary Methods:

Materials

All chemicals were used as received unless otherwise stated. Bovine serum albumin (BSA) (lyophilized powder ≥96%) and glucose oxidase (GOx) from Aspergillus niger, carbon disulfide (99%), hexamethylenediamine (98%), 2-ethyl-1-hexanol (≥98%), N- ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (98%), p-tosyl chloride (**99%),** LiCl and dimethylacetamide(DMAc) were all purchased from Sigma-Aldrich. Horseradish peroxidase (HRP) was purchased from Alfa-Aesar. N-isopropylacrylamide (NIPAAm, Aldrich, 98 %) and 2,2'-Azobis-(isobutyronitrile) (AIBN, Sigma, 98 %) were recrystallized twice in hexane and methanol respectively, prior to use. BS(PEG)9 (PEGylated bis(sulfosuccinimidyl)suberate) (Mw 708 g mol^{-1}, Thermofisher) was dissolved in anhydrous dimethyl sulfoxide (Sigma, ≥99.9%) and stored in argon or nitrogen gas at -20°C. The HEPES buffer comprised 50 mM HEPES, 10 mM $MgCl_2$, 100 mM KCl was adjusted to pH 7.6 with KOH.

RAFT agent synthesis

The synthesis of bis(propylsulfanylthiocarbonyl) disulfide was performed according to literature methods, with minor modificatons[1]. 1-propanethiol (15 g, 0.2 mol) was added dropwise to a 70 mL solution of KOH (14 g, 0.25 mol), followed by the addition of CS_2 (15 g, 0.2 mol) in two portions. This solution was first vigorously stirred at 25 ˚C for 30 min then cooled down in an ice bath. Subsequently, a solution of p-tosyl chloride (19 g, 0.1 mol) dissolved in acetone (100 mL) was added in portions over 10 min, and stirring was continued for 2 h. The acetone was evaporated. Afterwards, a red oil was extracted with dichloromethane; the resulting organic layer was washed multiple times with water and finally dried overnight with anhydrous $MgSO_4$, which was removed by filtration. Finally, dichloromethane was removed by rotary evaporation to yield bis(propylsulfanylthiocarbonyl) disulfide as a red viscous oil.

To synthesise the RAFT agent, 1.76 g of bis(propylsulfanylthiocarbonyl) and 2.27 g of ACVA-ACPM was dissolved in 50 mL of ethyl acetate and then refluxed for 18 hours. The solvent was evaporated to approximately 20 ml before isolating the product by column chromatography using silica gel as the stationary phase and ethyl acetate/hexane (50:50) as the eluent. Thin-layer chromatography (TLC) was used to guide the recovery of the product. Ethyl acetate and hexane were removed by evaporation and the sample then placed under vacuum overnight. The final product was an orange/red oil, which was characterized by C-NMR and H-NMR (Figure S3, S4) using a BRUKER Ascend 400 spectrometer at 400 MHz.

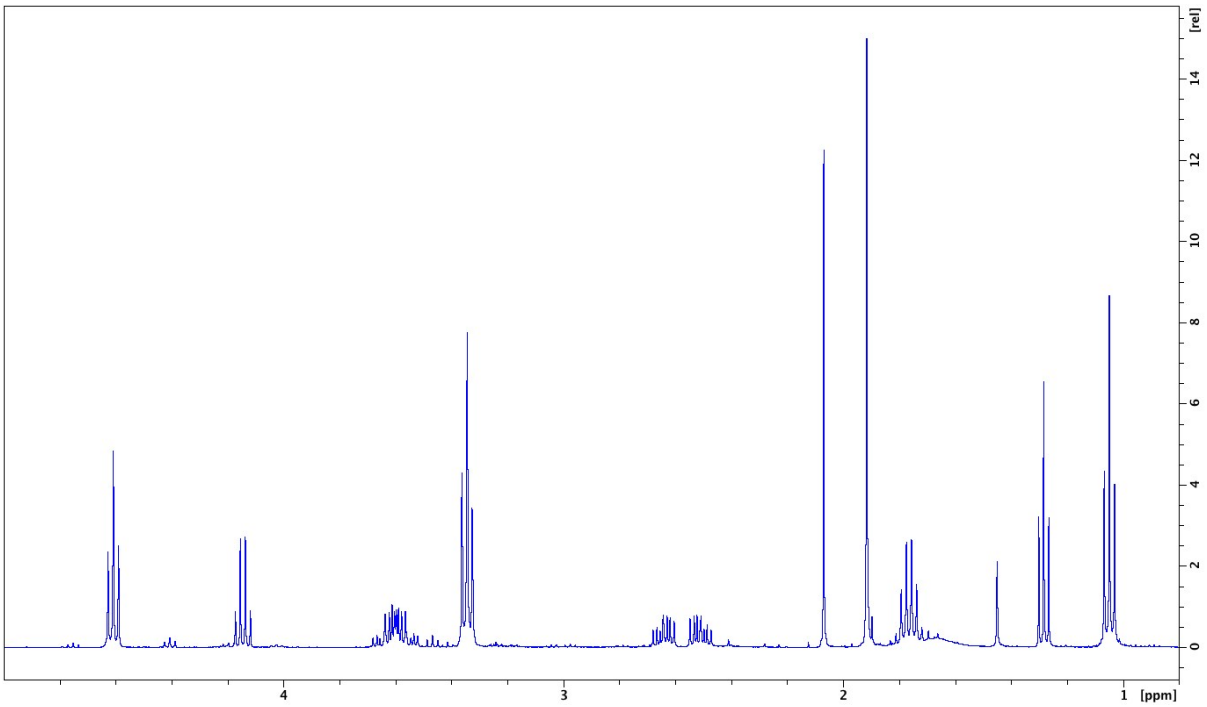

Figure S4: H NMR spectrum of mercaptothiazoline-activated trithiol-RAFT agent 4-cyano-4-(propyllsulfanylthiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide dissolved in $CDCl_3$.

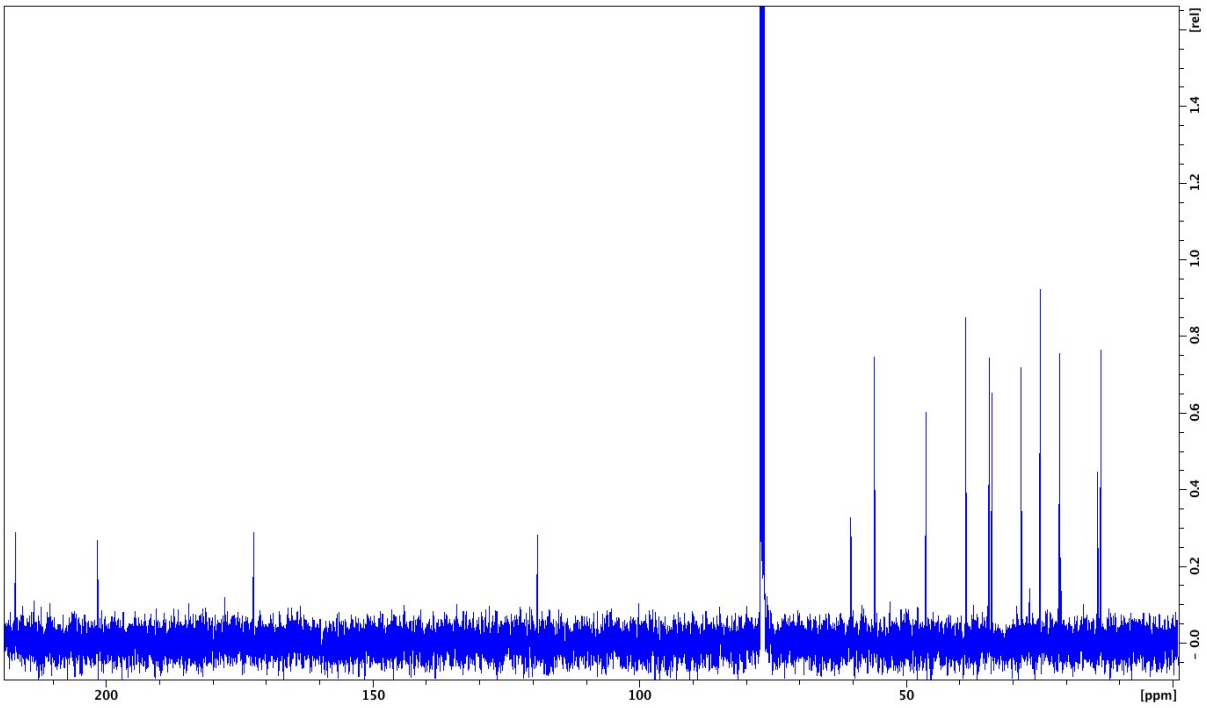

Figure S5: C NMR spectrum of mercaptothiazoline-activated trithiol-RAFT agent 4-cyano-4-(propyllsulfanylthiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide dissolved in $CDCl_3$

Polymerization of PNIPAAm

24 g of RAFT agent, 2 mg AIBN and 850 mg of NIPAAm were dissolved separately in acetonitrile, then combined (to a final volume 8 mL), placed into a 25-mL bottom round flask, sealed and degassed under argon for 40 minutes. The polymerization was carried out at 68°C for 8 hours. Acetonitrile was partially evaporated (c.a. 2 mL) in an open vessel. Subsequently, this solution was added drop wise to a 150-mL diethyl ether/hexane (2:1) (at -20°C) solution to precipitate the mercaptothiazoline-activated PNIPAAm. The diethyl ether/ hexane/acetonitrile solution was kept at -20°C for 10 min to maximize precipitation. The polymer was washed three times by repeated cycles of centrifugation at 4°C for 20 minutes, removal of the supernatant and addition of diethyl ether/ hexane (2:1). Finally, the PNIPAAm was dried overnight in vacuum to yield a yellow powder. Gel Permeation Chromotography (GPC LC 1100 v. Agilent Technologies with an RI-Detector and 2 Zorbax PSM Trimodal-S columns) was used to determine the number average molecular weight (Mn) of PNIPAAm (12000 g/mol) and its polydispersity index (PDI) (1.5. Mn). 2% vol dimethylacetamide (DMAc) in water and 3g/L of LiCl were flowed at a rate of 0.5 mL/min to elute the polymer. Pol(2-vinylpyridin) was used as a standard at 2 mg/mL after filtration through a 0.2 μm filter.

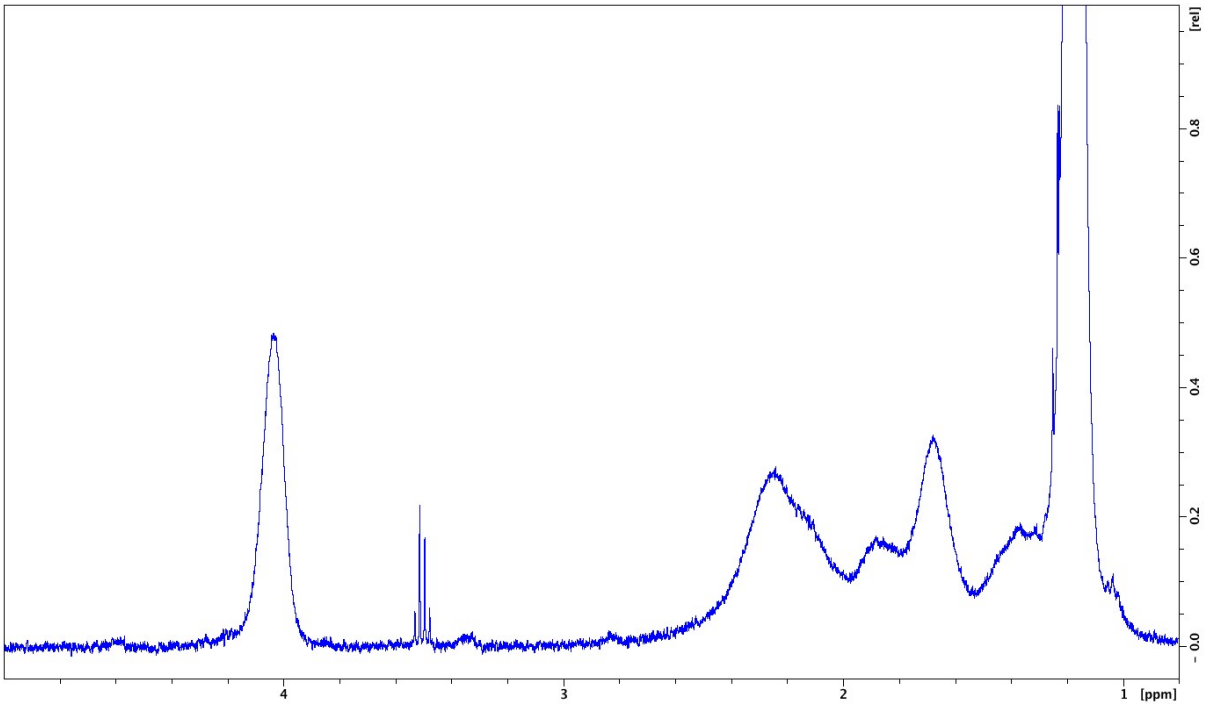

Figure S6: H NMR spectrum of mercaptothiazoline-activated PNIPAAm dissolved in $CDCl_3$.

Protein cationization and conjugation

Protein–polymer conjugates were synthesized as previously reported.[2] Aqueous solutions of either native BSA (10mg/ml) or native Glucose oxidase (10 mg/ml) was added to a solution of 1,6-diaminohexane (1.5 g in *c.a.* 10 mls) and the pH was adjusted to pH 6.2 using HCl. 100 mgs of EDAC were added to the solution and stirred for 3 hrs at room temperature before an additional 100 mg of

EDAC were added, the pH readjusted to pH 6.2 using HCl and the solution was left to stir overnight at room temperature. The solution was filtered with a 0.22-µm (MIllex GV syringe filter) to remove any precipitates and washed at least four times with Milli-Q water by ultrafiltration (Amicon® Ultra 15 mL filter with 30 kDa membrane) at 4°C and 4000 RCF. The cationized enzyme (BSA-NH_2 or GOx-NH_2) was lyophilized and stored at -20°C.

Device design and fabrication

Microfluidic devices were designed in AutoCAD (Autodesk, Switzerland). Master molds were fabricated using standard photolithography, and involved spin-coating two layers of SU-8 2010 photoresist (Microchem, USA) onto a silicon wafer (Silicon Materials, Germany) to yield a total height of 40 µm. All masters were treated with chlorotrimethylsilane (Sigma Aldrich, Switzerland) vapor in a vacuum desiccator to prevent adhesion of PDMS during molding and demolding.

Microfluidic devices were fabricated by pouring a 4 mm thick layer of polydimethylsiloxane (PDMS, Sylgard 184 A: B, 10:1, Dow Corning) onto the appropriate master. The PDMS was cured for approximately 24 hours at 70^{o}C, peeled off the master and Gauge 19 access holes punched. Devices for the production of proteinosomes were then bonded to a thin layer of PDMS after air plasma treatment. Lastly, all devices were bonded to a glass slide (24x75 mm, ThermoScientific, Switzerland). The assembled device was placed in an oven at 70^{o}C, overnight, to ensure complete bonding.

Determination of the number of droplets produced per minute

A video of 200 frames at a speed of 730 frames per second was acquired. Droplets were counted manually over a 274 ms period. The number of droplets was then multiplied by the duration of experiment to yield the exact number of droplets produced in each experiment.

Glucose Oxidase/ HRP assay:

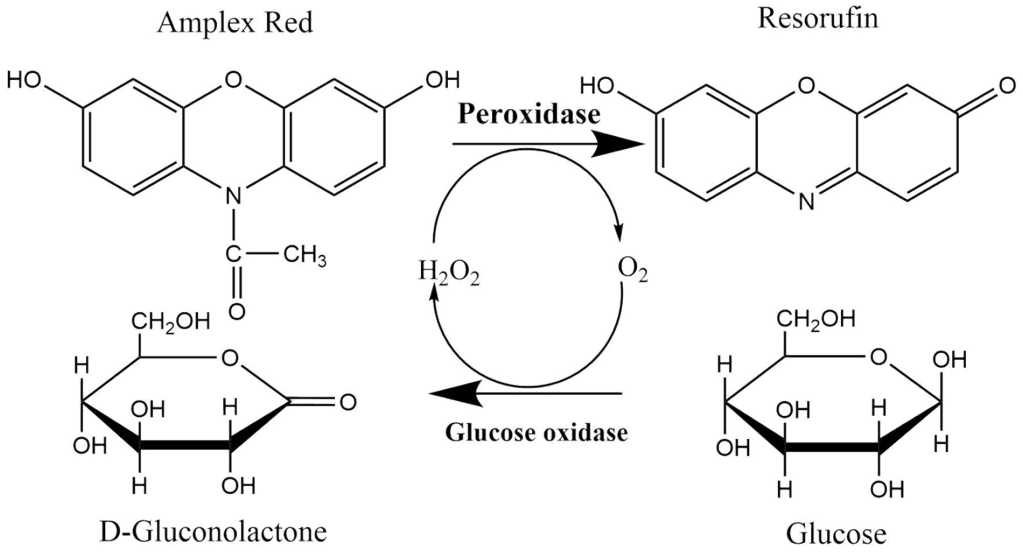

Figure S7: Schematic of Glucose oxidase/ HRP assay. Glucose is oxidised by glucose oxidase producing hydrogen peroxide as a side product. Turnover of hydrogen peroxide by the peroxidase in the presence of Amplex Red produces resorufin, which emits with a λ_{max} of 587 nm). Fluorescence emission is measured using a TECAN SPARK 20M well plate reader.

To determine the mass ratio of native GOx: HRP where HRP would be in excess, the reaction rate with increasing HRP concentration and constant GOx (0.025 mg/ml) was determined using 30- μM of glucose (Figure S7). Plotting the reaction rate as a function of GOx: HRP molar ratio showed that at a mass ratio of HRP:GOX of 0.08 the rate limiting step is the conversion of glucose to gluconolactone. Therefore, the molar ratio for HRP encapsulation within GOx-NH_2/PNIPAAm proteinosomes was fixed at 0.6 mg/mL and 4 mg/mL respectively, corresponding to a mass ratio of HRP:GOx of 0.15:1. The final concentration of conjugated and crosslinked GOx of the water-in-water proteinosomes was determined by absorbance at 280 nm ($E^{1\%}$= 16.7).

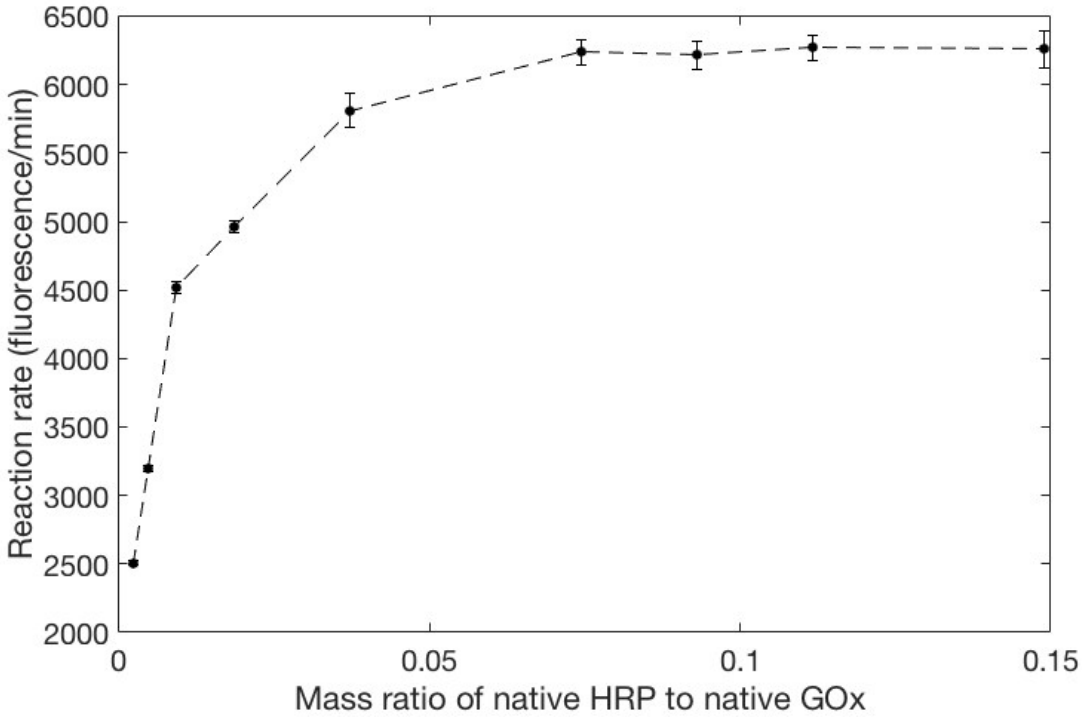

Figure S8: Plot of the reaction rate of varying native HRP mass to native GOx mass in the presence of 0.1 mM Amplex red for 30 μm glucose in HEPES buffer at 27 ^{o}C. Data show that at a mass ratio of HRP: GOx of 0.08:1, HRP is in excess and therefore the turnover of glucose oxidase to glucolactone is the rate limiting step. This was used to determine the mass ratio of HRP: GOx-NH_2/PNIPAAm mass ratio for proteinosome preparation.

References

1. Xu, X., Smith, A. E., Kirkland, S. E. & McCormick, C. L. Aqueous RAFT Synthesis of pH-Responsive Triblock Copolymer mPEO−PAPMA−PDPAEMA and Formation of Shell Cross-Linked Micelles †. *Macromolecules* **41,** 8429–8435 (2008).
2. Huang, X. *et al.* Interfacial assembly of protein–polymer nano-conjugates into stimulus-responsive biomimetic protocells. *Nat. Commun.* **4,** (2013).