Electronic Supplementary Information for:

"Mimicking Cellular Compartmentalization in a Hierarchical Protocell through Spontaneous Spatial Organization"

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1. Materials and Methods

1.1 Materials

All chemicals were used as received unless otherwise stated. For the synthesis of terpolymer: monomethoxy poly(ethylene glycol) 1 and 2 kDa was purchased from Rapp Polymere, trimethylene carbonate was purchased from TCI Europe. For the preparation of modified amylose derivatives: amylose (12-16 kDa) was supplied by Carbosynth and 3-chloro-2-hydroxypropyltrimethyl ammonium chloride (65 wt% in water) was supplied by TCI Europe. Dialysis Membranes MWCO 1000 KDa Spectra/Por® were used for protein purification. 10-acetyl-3,7-dihydroxyphenoxazine was purchased from Sanbio. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS, 1×, pH 7.4), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, cell membrane marker (Wheat germ agglutinin, Alexa Fluor 488 conjugate) and cell nuclear marker (Hoechst 33342) were obtained from ThermoFisher. Vascular cell basal medium (VCBM) and Endothelial Cell Growth Kit-VEGF were purchased from LGC standards. All other chemicals and reagents were supplied by Sigma-Aldrich. No unexpected or unusually high safety hazards were encountered with these materials or the following methods.

1.2 Synthesis

1.2.1 **PEG2$_{22}$-*b*-PCL$_{38}$gPTMC$_{32}$ synthesis**

This block copolymer was synthesized according to a previously reported literature procedure.[1] Briefly, monomethoxy-PEG-OH macroinitiator (705.2 mg) and stoichiometric amounts of ε-caprolactone (ε-CL) (2736 µL) and trimethylene carbonate (TMC) (2586 mg) were weighed in a flame-dried round bottom flask. Dry toluene (*ca*. 50 mL) was then added to the flask and evaporated in order to dry the contents before polymerization. Under argon, the dried reagents were re-dissolved in dry DCM (150 mL) and methanesulfonic acid (MSA) was added (0.1 equivalent with respect to ε-CL = 160 µL). The reaction was stored at RT for approx. 24 hours, until there was no evidence of residual monomer from the ^{1}H-NMR spectra. After completion was confirmed by ^{1}H-NMR, the reaction mixture was diluted using DCM and washed twice with saturated $NaHCO_3$ and once with brine before drying with Na_2SO_4, filtering and evaporating most of the solvent. The concentrated copolymer solution (in DCM) was then precipitated into ice cold diethyl ether (750 mL) and the remaining wax was partially dried under nitrogen before dissolving in dioxane and lyophilisation to yield a wax of 92 % yield, with Đ of 1.15. Both purity and copolymer composition was confirmed using ^{1}H NMR (Figure S1).

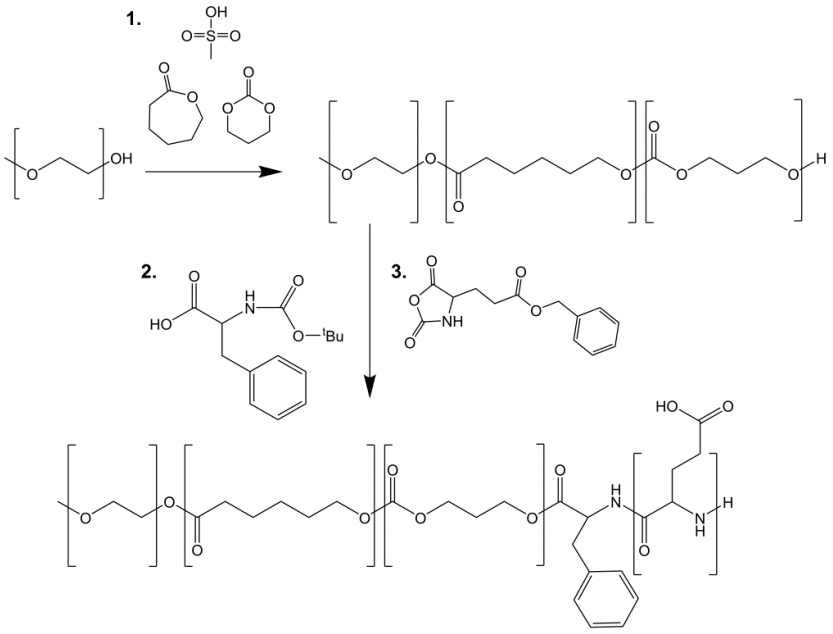
1.2.2 **PEG-*b*-PCLgPTMC-*b*-PGA terpolymer**[2]

Step 1 - Preparation of poly(ethylene glycol)-poly(caprolactone-*gradient*-trimethylene carbonate) (PEG-PCl$_g$TMC). According to a modified literature procedure the organocatalyzed ring-opening polymerization of ε-caprolactone and trimethylene carbonate was performed, aiming for a composition of PEG$_{44}$-PCl$_{50}$-*g*-TMC$_{50}$.[3] Monomethoxy-PEG-OH macroinitiator (2 kDa, 0.2 mmol, 400 mg) was weighed into a round- bottomed flask along ε-caprolactone (ε-CL, 10 mmol, 1108 µL) and trimethylene carbonate (TMC, 10 mmol, 1121 mg) and dried *via* azeotropic evaporation of added toluene (x3). The dried reagents were then re-dissolved in dry toluene (20 mL) and methanesulfonic acid (0.6 mmol, 39 uL) was added, under argon. The reaction mixture was stirred at 30 °C for 6 hrs, after which time it was precipitated into ice cold methanol. This yielded 2.1 g of a waxy solid (85 % yield) and the composition of the resulting copolymer was confirmed by ^{1}H NMR, comparing the protons of PEG (3.65-3.7 ppm), terminal methyl unit (singlet at 3.40 ppm) to PCL CH_2 (multiplet at 2.40-2.25 ppm) and PTMC CH_2 (multiplet at 2.2-1.8 ppm). GPC analysis (using a PL gel 5 µm mixed D column, with THF and PS standards) yielded a Đ of 1.1.

Step 2 - Chain-end modification with Boc-L-phenylalanine and deprotection. For the incorporation of a terminal amine onto the diblock copolymer, we performed a carbodiimide-mediated esterification between PEG-PCl*g*TMC and Boc-L-phenylalanine (Boc-L-Phe). 1.3 g (*ca*. 0.1 mmol) of PEG-PCl*g*TMC copolymer was dissolved in acetonitrile and to it was added dicyclohexylcarbodiimide (0.2 mmol, 41.9 mg), Boc-L-Phe (0.5 mmol, 23.4 mg), and 4-dimethylaminopyridine (0.02 mmol, 2.5 mg) on ice. The reaction mixture was stirred for 24 hrs at RT and afterwards placed directly in the freezer overnight to facilitate precipitation of dicyclohexylurea by-product. After cold filtration of the reaction mixture it was concentrated and then precipitated into cold methanol. ^{1}H NMR was used to check the product to confirm removal of the terminal TMC signal at 1.92 ppm and emergence of Boc protons at 1.45 ppm before deprotection. The resulting copolymer was then dissolved in 5 mL DCM, to which 5 mL of trifluoro acetic acid (TFA) was added (on ice) and the mixture was allowed to warm to RT and stirred for 2 hr. After 2 hr the solvent was evaporated and the copolymer was washed with $NaHCO_3$, 1 M NaCl and brine before drying on $MgSO_4$, filtration and evaporation of the majority

of the solvent so that the copolymer could be precipitated from ice cold methanol. ^{1}H NMR clearly showed that the signal arising from the terminal TMC group had disappeared, due to addition of phenylalanine at the terminus, and aromatic protons were visible at around 7.2-7.3 ppm. GPC analysis before and after deprotection yielded a Đ of 1.1, indicating that TFA treatment did not facilitate copolymer hydrolysis.

Step 3 - Polymerization and deprotection of *N*-carboxyanhydride γ-benzyl L-glutamate (NCA-BLG). Following a published method for the controlled polymerization of NCA-BLG by lowering the temperature and maintaining a constant flow of N_2 in order to remove CO_2 by-product,[4] we aimed to add between 8 and 10 PBLG units onto the amine-terminus of the copolymer chains. 1 g of Phe-terminated copolymer was weighed into a Schlenk flask and dissolved with *ca*. 3 mL of dry DMF and cooled in an ice bath. To the cooled mixture 160 mg of NCA-BLG was added under Ar and the reaction was left under a constant flow of N_2 for 24 hrs. The product was precipitated into cold methanol and analysed by ^{1}H NMR to confirm the overall composition and, in particular, the presence of benzylic and aromatic protons at 5.0-5.2 and 7.1-7.4 ppm, respectively. Benzyl-protected terpolymer was dissolved in 10 mL THF and 10 mL of methanol was added before applying the H-Cube at 60 °C with 30 bar of H_2 pressure and a flow rate of 1 mL/min to facilitate hydrolysis of benzyl protecting groups. The product was concentrated, then precipitated into cold ether, and dissolved in dioxane before lyophilisation to yield a waxy solid, 0.9 g (85 % yield). ^{1}H NMR was used to confirm successful deprotection of the PBLG units, and GPC data indicated that the polydispersity didn't increase beyond 1.1 during this process.



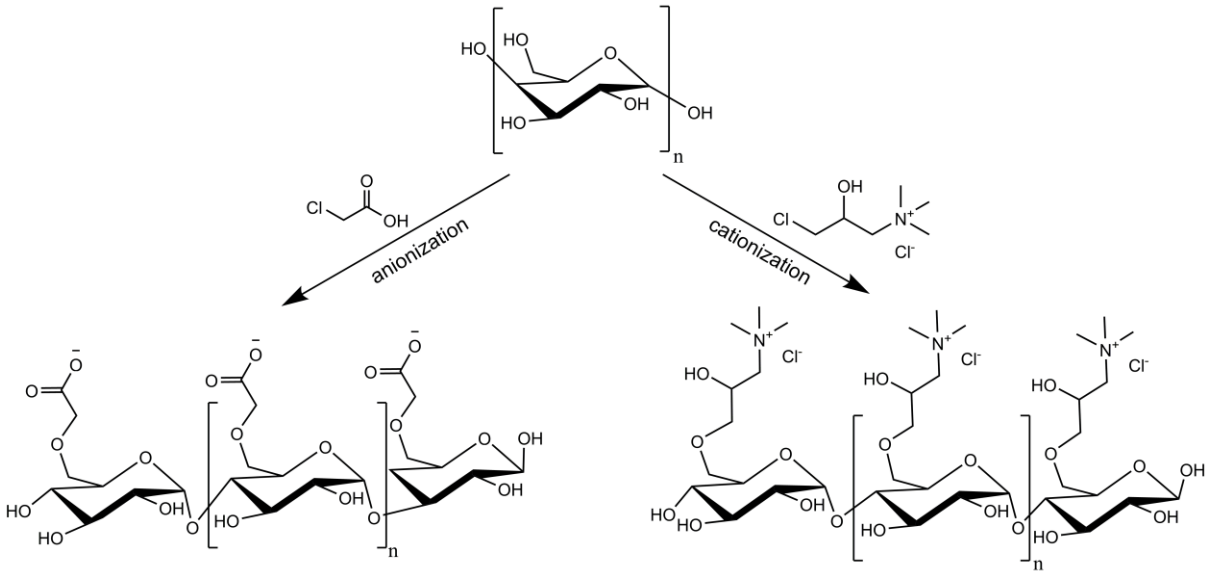
Scheme 1: Synthesis of PEG-*b*-PCL*g*PTMC-*b*-PGA terpolymer. Poly(ethylene glycol) monomethyl ether was used to initiate the ring opening polymerisation of ε-caprolactone and trimethylene carbonate (**step 1**). The terminal alcohol of this polymer was subsequently modified *via* a Steglich esterification with Boc-L-Phe-OH to yield a primary amine after TFA deprotection (**step 2**). The final poly(L-glutamic acid) block was introduced by the ring opening polymerisation of *N*-carboxyanhydride γ-benzyl L-glutamate, followed by hydrogenation (**step 3**)

1.2.3 **Modified amylose (Q-Am and CM-Am)**

Both quaternized (Q-Am) and carboxymethylated (CM-Am) were prepared in accordance with previously published procedures.[2]

Q-Am was prepared by dissolving 1.5g of amylose and 2.78g of NaOH in 14.25 mL of Milli-Q at 35 °C. After complete dissolution of the amylose, 11.64 mL of 3-chloro-2-hydroxypropyltrimethylammonium chloride solution (60 wt% in water) was added dropwise into the stirring reaction mixture, which was subsequently left to react overnight. After this time, the mixture was neutralized with acetic acid and precipitated into 200 mL of cold ethanol. The resulting precipitate was re-dissolved in Milli-Q water and dialysed extensively against water using regenerated cellulose dialysis tubing (Spectrum Labs, USA) with a 3.5 kDa MWCO before lyophilization. Around 5 g of Q-Am was obtained from this reaction (*ca*. 80 % yield). ^{1}H NMR characterization data presented in Figure S2a.

CM-Am was prepared by dissolving 1.5g of amylose and 3.6 g of NaOH in 15 mL of Milli-Q at 70 °C. After complete dissolution of the amylose, 2.7 g of chloroacetic acid was added and the reaction mixture was left to stir for 2 hours. After the reaction, the mixture was neutralized with acetic acid and precipitated into 200 mL of cold ethanol. The resulting precipitate was re-dissolved in Milli-Q water and dialysed extensively against water using regenerated cellulose dialysis tubing (Spectrum Labs, USA) with a 3.5 kDa MWCO before lyophilization. Around 5 g of CM-Am was obtained from this reaction (*ca*. 80 % yield). Around 3 g of CM-Am was obtained from this reaction (*ca*. 90 % yield). ^{1}H NMR characterization data are presented in Figure S2b.



Scheme 2: Modification of amylose with either chloroacetic acid or 3-chloro-2-hydroxypropyltrimethylammonium chloride solution to form CM-Am and Q-Am, respectively.

1.2.4 **Synthesis of dye-labelled bovine serum albumin**

Bovine serum albumin (BSA) was dissolved at a concentration of 2 mg/ml in 100 mM $NaHCO_3$ (adjusted to pH 9) in a 1.5 mL Eppendorf tube. To this solution was added a solution of an amine-reactive dye (10 mM, in DMSO, either fluorescein isothiocyanate, rhodamine isothiocyanate, or Cy5-NHS) at a 1:1 stoichiometry. For example, 2.3 mg of BSA (35 nmol) was dissolved in 1.15 mL of buffer, followed by the addition of 3.5 µL of a 10 mM stock of FITC

in DMSO (35 nmol). The reaction mixture was then mixed, in the dark, at room temperature for 18 hours. It was then concentrated to approximately 200 µL and purified *via* strong anion exchange chromatography (HiTrap Q HP, GE Life Sciences) using a gradient from 10 mM $NaHPO_4$, pH 7 to 10 mM $NaHPO_4$, 1 M NaCl, pH 7 to ensure removal of free dye. Conjugate-containing fractions were pooled, concentrated, and adjusted to approximately 1 mg/mL using a NanoDrop to measure the concentration. The dye functionalization efficiencies were calculated from the UV-Vis spectra of bioconjugates. BSA-FITC (489 nm): 0.46 FITC per BSA. BSA-Cy5 (670 nm): 0.27 Cy5 per BSA. BSA-RITC (555 nm): 0.30 RITC per BSA.

1.2.5 Synthesis of Protease K substrate

BSA (30 mg) was dissolved at a concentration of 10 mg/ml in 100 mM $NaHCO_3$ (adjusted to pH 9). An excess of FITC (6 mg) was subsequently added and the mixture left to react overnight at room temperature in the dark. The crude reaction mixture was purified *via* a sephadex G25 desalting column prior to lyophilisation.

1.2.6 Synthesis of succinylated enzymes

Glucose oxidase (GOX) or horseradish peroxidase (HRP) was dissolved at a concentration of 2 mg/ml in 100 mM $NaHCO_3$ (adjusted to pH 9) in a 1.5 mL Eppendorf tube. To this solution succinic anhydride was added directly to a concentration of 50 mM (for example, 5 mg solid succinic anhydride was added to 1 mL of reaction mixture). The reaction mixture was mixed for 4 hours before it was concentrated to approximately 200 µL and purified *via* strong anion exchange chromatography (HiTrap Q HP, GE Life Sciences) using a gradient from 10 mM $NaHPO_4$, pH 7 to 10 mM $NaHPO_4$, 1 M NaCl, pH 7. Protein containing fractions were pooled, concentrated, and stored at -20 °C until further use. The degree of succinylation was not determined.

1.2.7 Recombinant super folder GFP expression and purification

BL21 (DE3) cells were transformed with a pET28a vector containing a super folder green fluorescent protein (sfGFP) gene with an N-terminal histidine tag (made by EPOCH Life Science). A 1 L culture containing kanamycin (50 µg/mL final concentration) was inoculated with a 10 mL overnight culture of the transformed BL21 (DE3). The 1 L culture was grown to an OD_{600} ~ 1.5 in terrific broth at 37 °C, shaking at 180 rpm, before induction of protein expression by addition of IPTG (0.3 mM final concentration). Protein expression occurred for 20 h at 20 °C, 180 rpm. The cells were pelleted by centrifugation at 4000 xg for 10 min and pellet was resuspended in lysis buffer (20 mM HEPES, 150 mM NaCl, 20 mM imidazole), then lysed using a cell disruptor. The cell debris was removed by spinning the lysate at 60000 xg for 30 min. The supernatant containing soluble histidine-tagged sfGFP protein was purified using Ni-NTA column chromatography (HisTrap FF GE Healthcare) followed by a final polishing step using size exclusion chromatography (SEC 16/600 GE Healthcare). The protein purity was assessed using SDS-PAGE and identity was confirmed using Q-TOF mass spectrometry. The freshly purified protein, in PBS, pH 7.4, was flash frozen using liquid nitrogen for storage at - 80 °C.

1.3 Formation of self-assembled systems

1.3.1 General procedure for polymersome formation and enzyme encapsulation

In a 5 mL vial, 20 µL of PEG-*b*-P(CL-*g*-TMC) block-copolymer (10 % in PEG 350 *w/w*) was added. A magnetic stirring bar was introduced and a thin film of the polymer solution was

created by slow stirring. Subsequently, 80 μL of aqueous solution, with or without enzymes (>5 mg mL^{-1}), was directly added followed by continuous stirring for 5 min. Polymersomes were subsequently diluted and purified via dialysis.

1.3.2 General procedure for polymersome-in-coacervates formation

Q-Am and CM-Am were dissolved separately in phosphate-buffered saline at a concentration of 0.66 mg/ml. Coacervation was induced by mixing the solutions of Q-Am and CM-Am in a ratio of 2:1. Typically, 75 μL of CM-Am was added to a 1.5 mL Eppendorf tube containing 150 μL of Q-Am while stirring in an Eppendorf tube shaker at 1500 rpm and 25 °C. Immediately after coacervation was initiated, a solution containing polymersomes (75 μL, polymer concentration ca. 5 mg/ml) was added. After 4 minutes of stirring, 9 μL of terpolymer stock solution (50 mg/ml in DMSO) was slowly added to stabilise the coacervate droplets. The specific conditions for each experiment displayed in the figures are listed below:

Figure 1: FITC-BSA, RITC-BSA, and Cy5-BSA polymersomes mixed in equal parts prior to addition (75 μL) to the nascent coacervate droplets.

Figure 2: After encapsulation, protein concentrations in GOX, HRP, and HRP-GOX polymersomes were determined via a BCA assay. Samples were diluted in PBS (Table S3) to equalise their protein concentrations prior to addition to nascent coacervate droplets (75 μL). Samples were purified via gentle centrifugation (as described below) prior to plate reader assays.

Figure 3: Encapsulated ProtK was quantified via a BCA assay prior to addition to the nascent coacervate droplets. See S1.4.3 for more details.

Figure 4: Q-Am and CM-Am were dissolved separately at a concentration of 1 mg/mL in VCBM which was supplemented with Endothelial Cell Growth Kit-VEGF. Coacervation was induced by the addition of 100 μL CM-Am to 200 μL Q-Am whilst shaking at 1500 rpm. After 5 minutes of mixing, 9 μL 50 mg/mL terpolymer in DMSO was added, containing 1 v/v% 1 mg/mL Nile red in DMSO.

1.4 Physical characterisation of polymersomes

1.4.1 AF4 fractionation of empty- and filled-polymersomes

The asymmetric flow field flow fractionation static light scattering (AF4-SLS) experiments were performed using a Wyatt Dualtech AF4 instrument, equipped with a short channel. Regenerated cellulose (RC) 10 kDa membrane (Millipore) was used as membrane. A spacer of 350 μm was used. The following SLS detectors were connected to the AF4 system: a Wyatt DAWN HELEOS II light scattering detector (MALS), which was installed at various angles (12.9 °, 20.6 °, 29.6 °, 37.4 °, 44.8 °, 53.0 °, 61.1 °, 70.1 °, 80.1 °, 90.0 °, 99.9 °, 109.9 °, 120.1 °, 130.5 °, 149.1 ° and 157.8 °) operated with a 664.5 nm laser. Prior to the measurements SLS detectors were normalized using Bovine Serum Albumin (BSA). The AF4 channel was pre-washed with PBS. This solution was also used as eluent. The processing and analysis of the Rg data were performed using Astra 7.1.2. The exact method used for AF4 fractionation is available in Table S1.

Start (min)	End (min)	Mode	Cross flow start (mL min^{-1})	Cross flow end (mL min^{-1})
0	1	Elution	3.00	3.00
1	2	Focus	-	-
2	3	Focus + inject	-	-
3	6	Elution	3.00	1.17
6	8	Elution	1.17	0.49
8	10	Elution	0.49	0.24
10	13	Elution	0.24	0.10
13	30	Elution	0.10	0.10
30	31	Elution	0.00	0.00
31	32	Elution + inject	0.00	0.00
32	37	Elution	0.00	0.00

Table S1: General method for the AF4 fractionation of empty and filled polymersomes. The following flow conditions were applied: 1.5 mL min^{-1} detector flow, 1.00 mL min^{-1} focus flow and 0.20 mL min^{-1} injection flow. 7 µL from a 10 mg mL^{-1} polymersomes were injected into the AF4 SC.

1.4.2 **Zeta (ζ) potential measurements**

ζ measurements were performed on a Malvern instrument Zetasizer (model Nano ZSP). Zetasizer software was used to process and analyse the data.

Sample	Zeta potential (mV)
HRP: GOX	12.8 ± 7.4
HRP	13.9 ± 8.0
GOX	14.9 ± 8.1

Table S2: Zeta potentials of polymersomes containing encapsulated succinylated proteins as indicated

1.4.3 **Determination of concentrations of encapsulated proteins inside polymersomes**

The concentration of proteins encapsulated in polymersomes was determined by the Pierce™ BCA Protein Assay according to manufacturer's instructions. Polymersomes containing no cargo were used as a blank and the absorbance value was subtracted from the protein-filled polymersomes. Known concentrations of protein stock solutions (determined using the absorbance at 280 nm by the Nanodrop™) were measured and correlated to the BSA calibration curve in order to obtain a correction factor for each protein. Measurements were done in duplo, and standard deviation from the two measurements were calculated to give the error in Table S2.

Sample	Mean concentration (µg/mL)	Standard Deviation (µg/mL)	V(sample) used in dilution (µL)	V(PBS) used in dilution (µL)	Total protein (µg)
HRP: GOX	123.9	3.8	55	45	5.1
HRP	55.8	2.1	80	20*	3.4
GOX	115	15	20	80*	1.7

Table S3: Concentrations of encapsulated succinylated proteins as derived from BCA assay. *For the HRP + GOX separately encapsulated, 80 µL HRP polymersomes were diluted with 20 µL GOX polymersomes instead of PBS.

1.5 Coacervate manipulations

1.5.1 Coacervate purification procedure using centrifugation

Stabilised coacervates were centrifuged into a pellet at 500 xg for 4 minutes. The supernatant was pipetted away without disturbing the pellet and replaced with the same volume of fresh PBS. The pellet was gently resuspended in fresh buffer. Bright field microscopy was used to show that the coacervate structure is retained during this process (Figure S6).

1.5.2 Deriving sequestration efficiency using spectrophotometry

The fluorescence intensity of polymersomes with encapsulated cargo was determined for FITC-BSA (λex = 488 nm , λem = 515 nm) and sfGFP (λex = 488 nm, λem = 510 nm) using the Cary Eclipse Fluorescence Spectrophotometer (Varian). The fluorescence values were background subtracted using coacervate samples with no cargo loaded. The fluorescence values obtained were an average of at least two separate encapsulation experiments, and the error is the standard deviation between these values. For the sfGFP samples, the fluorescence of the purified coacervates was directly measured. However, for the succinylated BSA samples, only the supernatant fluorescence was measured, and the fluorescence 'after purification' was derived by subtracting the supernatant fluorescence from the fluorescence before purification (Table S4). The sequestration efficiency was determined using the following formula:

$$SE\%\ (spectrophotometer) = 100 \times \frac{Fluorescence\ (purified\ coacervates)}{Fluorescence\ (coacervates\ before\ purification)}$$

Sequestered cargo (% v/v of polymersome in coacervates)		Average fluorescence (a.u.)	Standard deviation (a.u.)
sfGFP (12%)	before purification	395.8	3.1
	after purification	12.2	2.7
sfGFP (24%)	before purification	703.1	5.8
	after purification	19.8	0.5
BSA-FITC (14%)	before purification	402.7	3.0
	supernatant	364.1	4.7
	calculated 'after purification'	*38.6*	
BSA-FITC (20%)	before purification	477.5	3.7
	supernatant	432.6	4.4
	calculated 'after purification'	*44.9*	

Table S4: The fluorescence values of coacervates before and after purification that were used to derive the sequestration efficiency.

1.5.3 Determining sequestration efficiency from confocal images

The average fluorescent emission values of fluorescent polymersomes were measured inside a number of coacervate protocells, and compared to the same area outside the coacervate

protocells. The absolute number of polymersomes outside coacervate protocells was approximated by multiplying this value by a factor of 100, to correct for the approximate 100-fold difference in volume between the condensed coacervate phase and the polymer-poor supernatant. This value of 100 is obtained by measuring the volume of polymer-rich phase (~1 µL) compared to the total solution volume (100 µL) of a coacervate sample formed in the same way as described above. The partitioning coefficient was thus calculated as:

$$EE\%\ (confocal\ images) = 100 \times \frac{Fluorescence\ inside\ protocells}{Fluorescence\ outside\ and\ inside\ protocells}$$

1.5.4 Incubation of protocells in cell media

Coacervates were prepared in VCBM as previously described and dialyzed against VCBM using a Float-A-Lyzer G2 dialysis device with a MWCO of 1000 kDa. The membrane of the dialysis device was pre-wetted according to the manufacturer's protocol using 70% ethanol and demineralized water. The dialysis device was conditioned by rinsing the inside of the membrane with VCBM. The coacervates were pipetted inside the device and dialyzed overnight at 4ºC against 50 mL VCBM. After dialysis, the coacervates were recovered from the bottom of the membrane, transferred to an ibidi 8 well chamber slide and imaged.

1.5.5 Co-incubation of protocells and HeLa

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FBS) and 1% penicillin/streptomycin at 37 ºC in a humidified atmosphere of 5% CO_2. To observe the interaction between HeLa cells and protocells, HeLa cells were seeded in µ-Slide 8 wells (Ibidi) using a standard trypsin-based technique. After culturing in DMEM medium for 24 h, PBS buffer was used to remove the DMEM medium. The cells were then stained with Alexa Fluor™ 488 conjugate of wheat germ agglutinin and Hoechst 33342 for 10 min, respectively. Thereafter, 20 µL of protocell suspension was added to the cells, followed by observation via confocal laser scanning microscopy (Leica TCS SP5X).

1.6 Confocal scanning laser microscopy

Protocells (100 µL) were deposited in an 8-well microscopy slide (cat. #80827, Ibidi, Germany). Images were acquired using a Leica TCS SP5 confocal laser scanning microscope equipped with diode lasers, using an HC PL APO CS2 63x/1.20 water immersion objective. The pinhole was set to 1 Airy Unit. To ensure the highest possible temporal resolution, a resonance scanning mode was engaged, enabling scan speeds of 12000 Hz, capable of acquiring a single 512x512 frame in 0.050 seconds. Frames were acquired sequentially to minimise crosstalk between channels. Images were acquired with the following parameters:

- FITC: λ_{ex} 488 nm (1% laser power); λ_{em} at 495 nm – 540 nm.
- RITC: λ_{ex} 552 nm (1.5% laser power); λ_{em} at 564 nm – 665 nm.
- Cy5: λ_{ex} 638 nm (2% laser power); λ_{em} at 642 nm – 780 nm.

Figure 4 imaging was performed on a Zeiss LSM510 META NLO confocal microscope equipped with a C-apochromat 63x N.A. 1.2 water immersion objective. Coacervate membranes were excited with a HeNe laser (543 nm) and emission was collected > 545 nm.

1.7 Enzymatic cascades

1.7.1 Determination of free protein concentration

Enzyme concentrations for free proteins was determined using the standard protein absorbance at 280 nm obtained by the Nanodrop™.

1.7.2 Reaction progress measurements for GOX & HRP in bulk

20 µL stabilised, purified coacervate sample was mixed with 130 µL reaction master mix containing amplex red (250 µM), and D-glucose (final concentrations of either 0 mM, 1mM, 5 mM or 25 mM as indicated in figures) at 25 °C in PBS, pH 7.4. The reaction was monitored using resorufin fluorescence (λex = 530 nm, λem = 590 nm) on the Spark ® 10M microplate reader (TECAN), and the fluorescence values were background subtracted. The error is the standard deviation of triplicates from at least two different experiments.

1.7.3 Confocal measurements

Protocells (100 µL) were deposited in an 8-well microscopy slide (cat. #80827, Ibidi, Germany) and allowed to settle for several minutes. In this time a suitable area to monitor the reaction was located. Once an area of interest was defined, 1 µl of 5 µM Amplex Red in DMSO was gently pipetted into the well, without moving the sample. To start the cascade, 1 µl of 1 M glucose was gently pipetted into the sample and a time series was commenced immediately.

Images were acquired using a Leica TCS SP5 confocal laser scanning microscope equipped with a diode laser, using an HC PL APO CS2 63x/1.20 water immersion objective. The pinhole was set to 1 Airy Unit (111.5 µm). Time lapse images were recorded at 20 fps, starting right after glucose addition. Images were acquired with the following parameters: 512x512 size, 12000 Hz scan rate, excitation at 552 nm (0.1% laser power); emission at 564 nm – 665 nm.

1.7.4 Artificial lysosome spectrophotometer measurements

The fluorescence of BSA-FITC (λ_{ex} = 488 nm, λ_{em} = 515 nm) was measured in quartz cuvettes using the Cary Eclipse Fluorescence Spectrophotometer (Varian). For the artificial lysosome sample, 75 µL proteinase K encapsulated in polymersomes (30 µg/mL protein according to BCA assay, so final protein concentration is 7.2 µg/mL) were mixed with BSA-FITC (50 µg/mL final concentration) and 225 µL coacervate (Q:CM in a 2:1 at 0.6 mg/mL each) prior to stabilisation with 9 µL terpolymer (50 mg/mL in DMSO) after 7 min of shaking at 1500 rpm. For the free proteinase K and BSA-FITC control, the proteins were mixed to a final concentration of 7.2 µg/mL and 50 µg/mL respectively, and the reaction monitored immediately. For the encapsulated BSA-FITC sample, BSA-FITC (50 µg/mL final concentration) and 225 µL coacervate (Q:CM in a 2:1 at 0.6 mg/mL each) prior to stabilisation with 9 µL terpolymer (50 mg/mL in DMSO) after 7 min of shaking at 1500 rpm. The coacervates were purified using centrifugation and fluorescence of the resuspended pellet was monitored. Free proteinase K protein was added to the protected BSA-FITC loaded coacervates after 54 s. The fluorescence was monitored for 180 s before SDS (5% w/v final) was added to disrupt the compartmentalisation. The data was normalised as a whole using feature scaling to bring the fluorescence values between 0 and 1. Results in Figure 4 are an average of triplicate data from a round of sample preparation. For each sample, experiments were performed at least twice to check reproducibility of results.

2. Supplementary Figures

Figure S1: PEG-b-PCLgPTMC NMR characterization

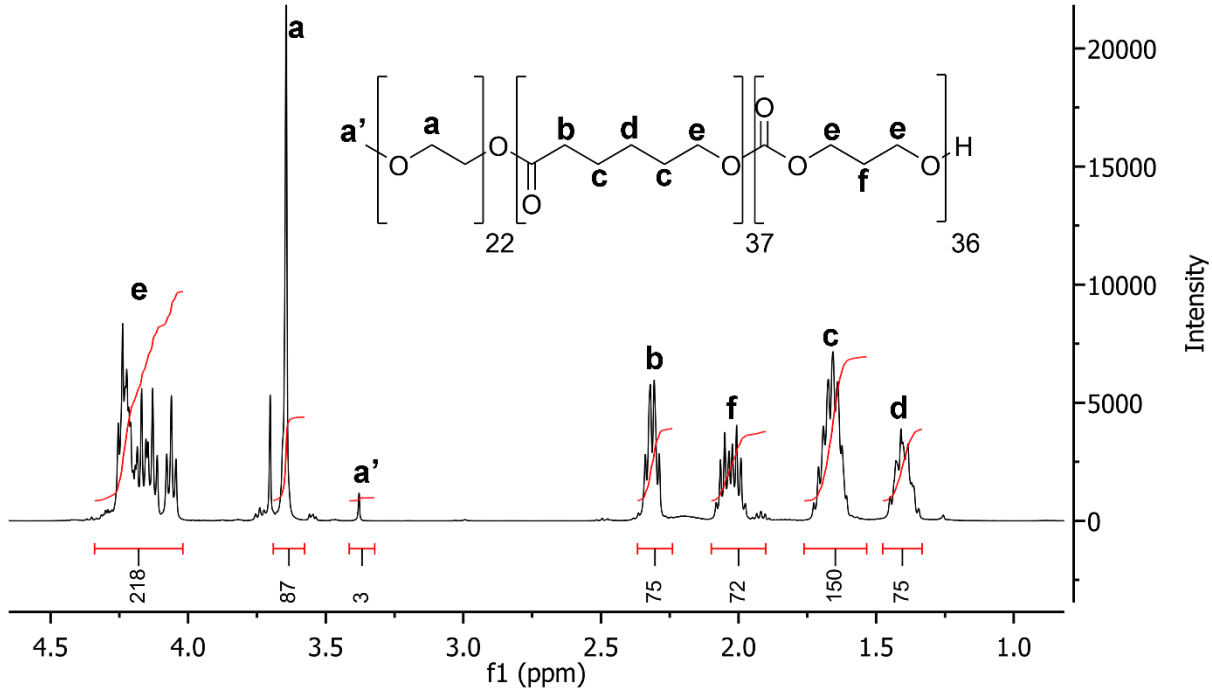


Figure S1: ^{1}H NMR spectrum of PEG-*b*-PCL*g*PTMC.

Figure S2: Amylose characterisation

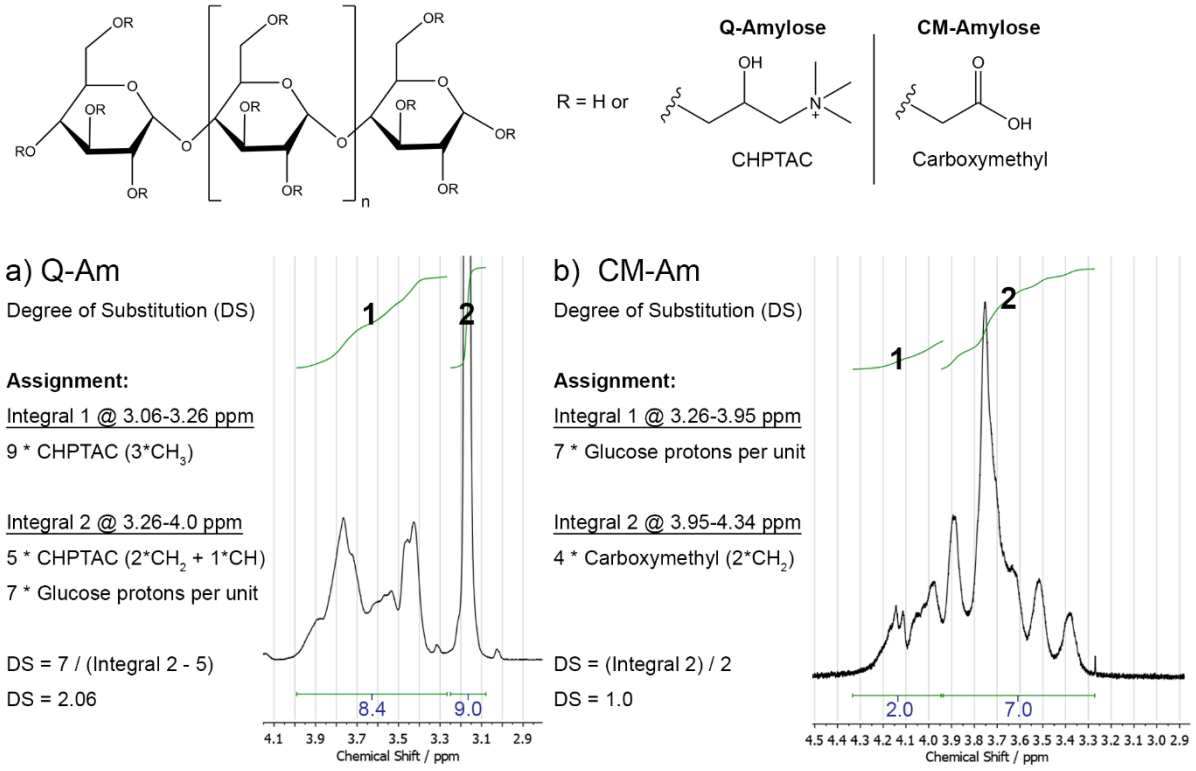


Figure S2: ^{1}H NMR processing of highly charged amylose derivatives, including calculation of the degree of substitution (DS = number of modifying groups per glucose unit), of both **a.** quaternized (Q-Am) and **b.** carboxymethylated (CM-Am) products.

Figure S3: Polymersome characterisation

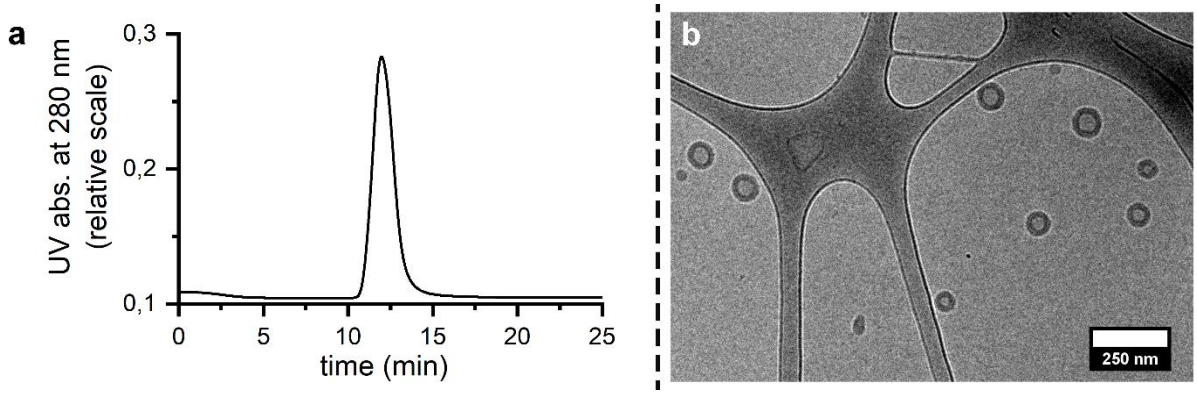


Figure S3: **A**. Chromatogram of BSA-filled polymersomes after purification, indicating the absence of non-encapsulated protein. **B.** Cryo-TEM image of PEG-PCLgPTMC polymersomes.

Figure S4: Sequestration efficiency (SE) of polymersomes inside coacervates

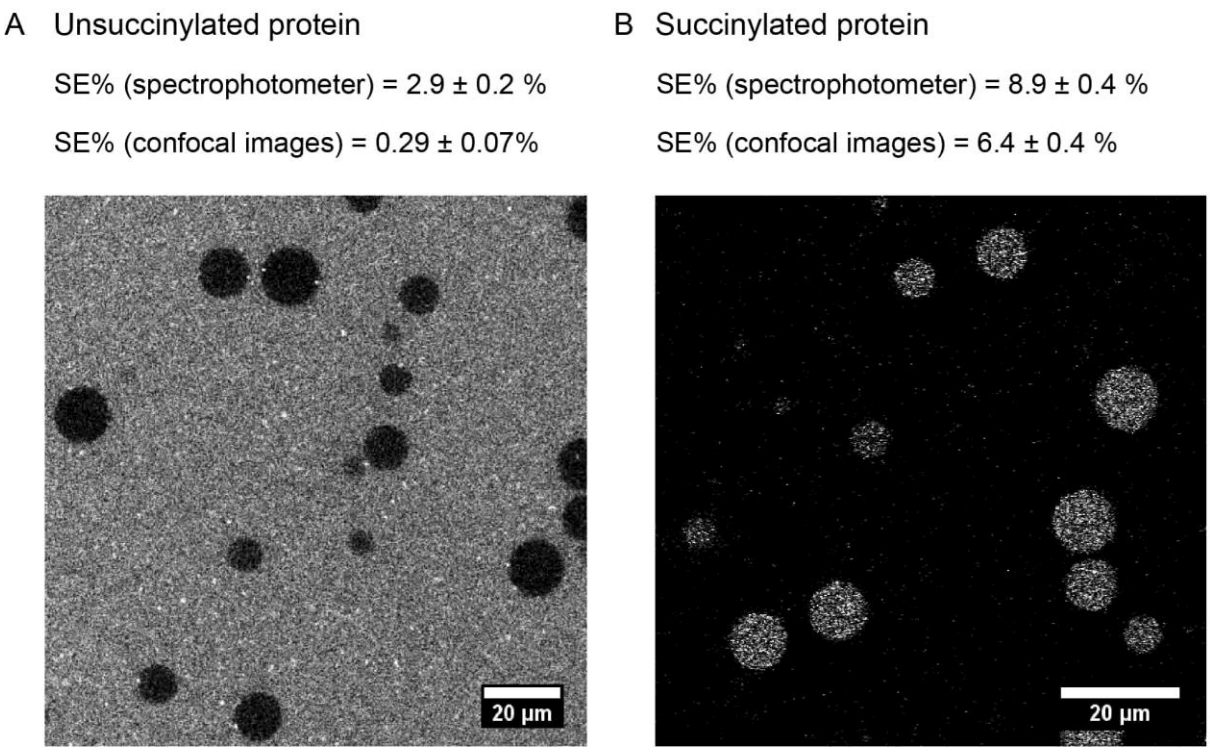


Figure S4: The differing sequestration efficiency (SE) of unsuccinylated sfGFP (**A**), and succinylated FITC-BSA (**B**). The confocal method relies on the volume of the polymer-rich phase, which is approximated to be 1 µL. It is difficult to measure smaller volumes than this, and if the polymer-rich phase is indeed smaller than approximated, the estimated SE% would be slightly higher. In comparison, the spectrophotometric method relies on accurate pipetting of the supernatant, and errors can be introduced if the pellet is difficult to locate by eye, if accidental removal of some coacervates occurs, or if it only considers larger coacervates. For example, if any residual supernatant is not removed, it could give a higher than expected read out.

Figure S5: Polymersome characterisation

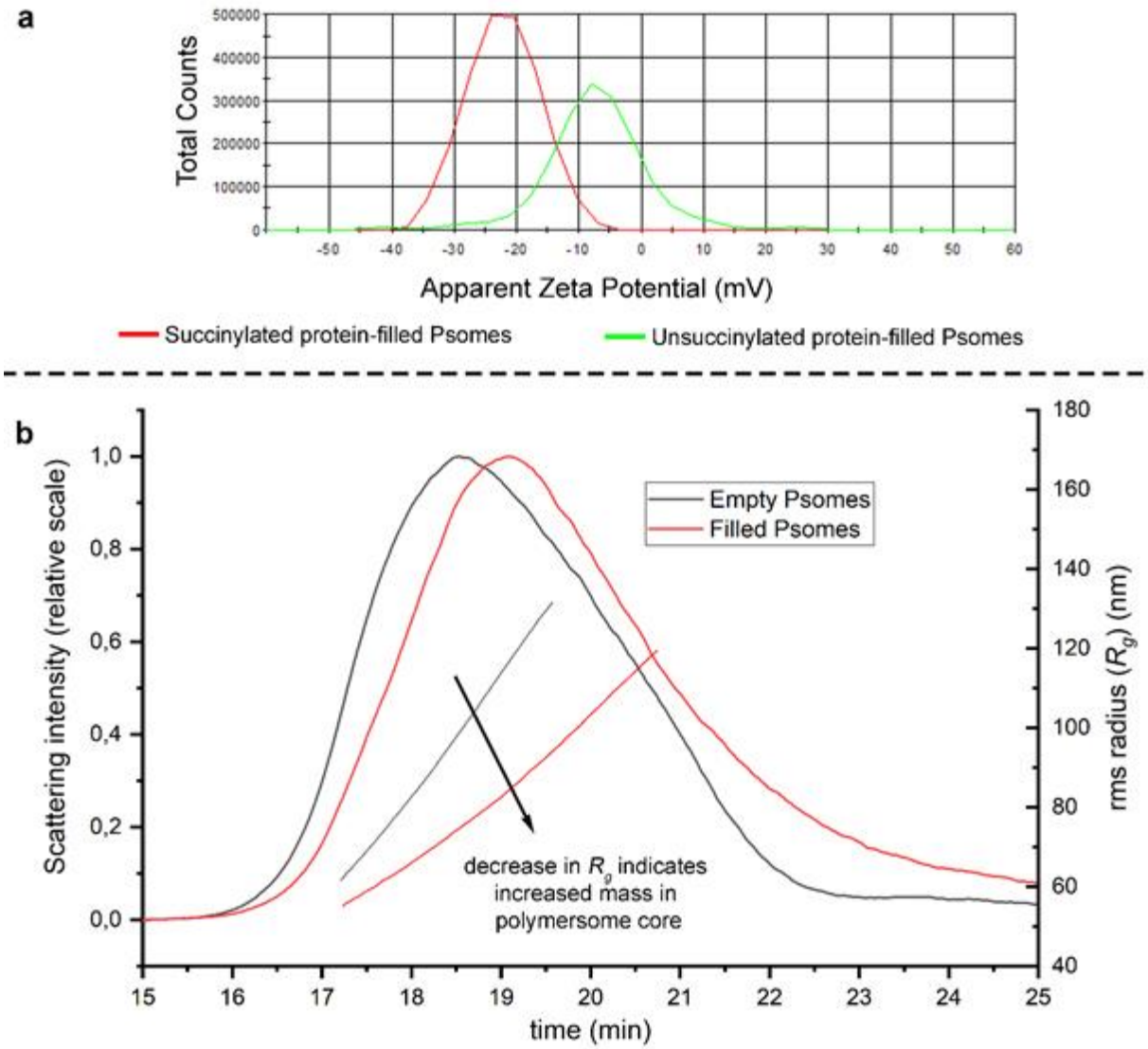
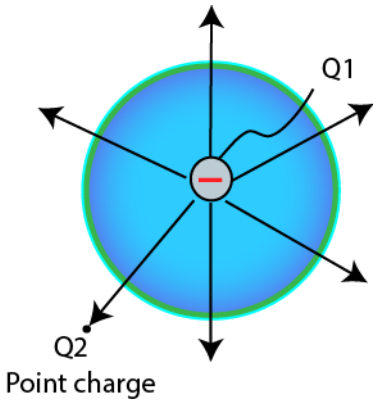


Figure S5: **A.** AF4 fractograms of empty and protein-filled polymersomes. A decrease in the measured radius of gyration (R_g) correlates to increased mass in the core of each particle, from encapsulated protein. **B.** Zeta potential distribution of polymersomes encapsulating either succinylated or non-succinylated BSA.

Figure S6: Theoretical approximation of the increase in electric field generated by charged cargo following the Law of Coloumb's Potential:



$$E = \frac{F}{Q2} = K\frac{\frac{Q1\,Q2}{r^2}}{Q2} = K\left(\frac{Q1}{r^2}\right)$$

Figure S6: Where *E* is the magnitude of the electric field at certain point of space, *F* is the electric force generated from Q1 (charge of cargo), *K* is the electric constant and is equal to 9 × 10^9 Nm^2/C^2, r is the center to center distance (i.e. distance between encapsulated proteins and the surface of the polymersome).

In one succinylated BSA, approximately 59 electrons (where one electron charge is 1.6021765 x 10^{-19} C) are available; therefore, the magnitude of the electric charge created at the polymersomes surface will be:

$$E = 9 \times 10^9 \frac{\mathrm{N}m^2}{C^2} \times \frac{(59 \times 1.6021765 \times 10^{-19})C}{(80 \times 10^{-9})^2\, m^2} = 1.33 \times 10^7\, N/C$$

Figure S7: Validation of protocell purification protocol

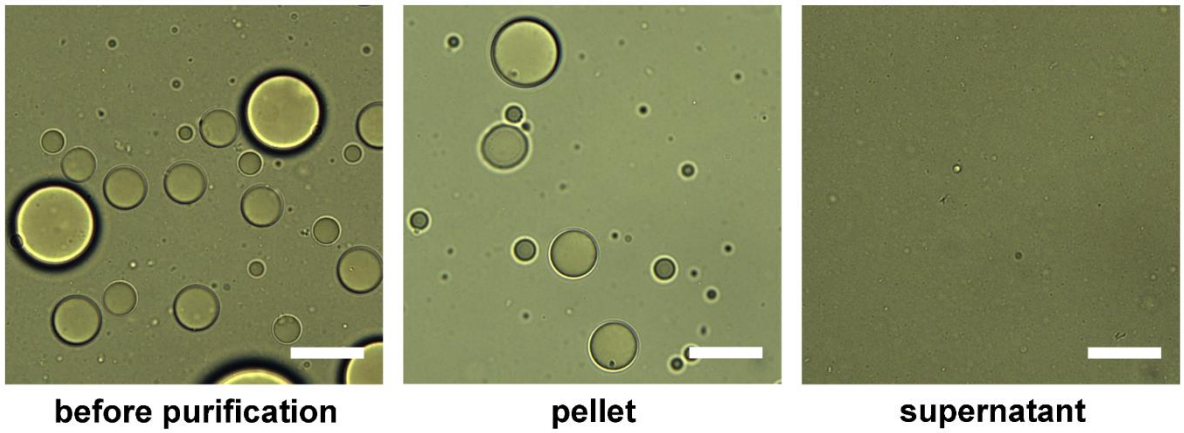


Figure S7: Brightfield images showing coacervates remaining intact before and after purification. Scale bars represent 20 µm.

Figure S8: Importance of terpolymer membrane

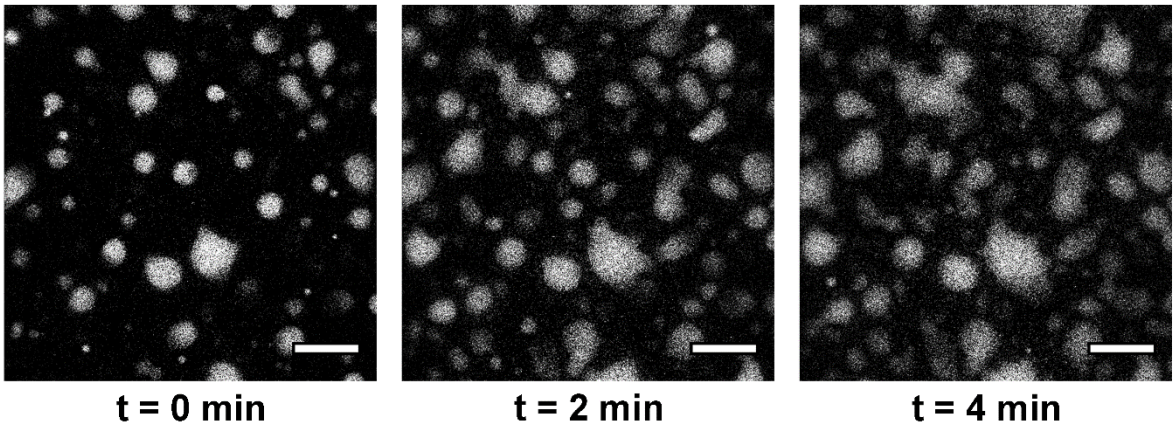


Figure S8: Confocal micrograph of FITC-BSA taken up by coacervate microdroplets, without terpolymer stabilisation. Scale bar represents 50 µm.

Figure S9: Uniform spatial organisation of polymersomal organelles

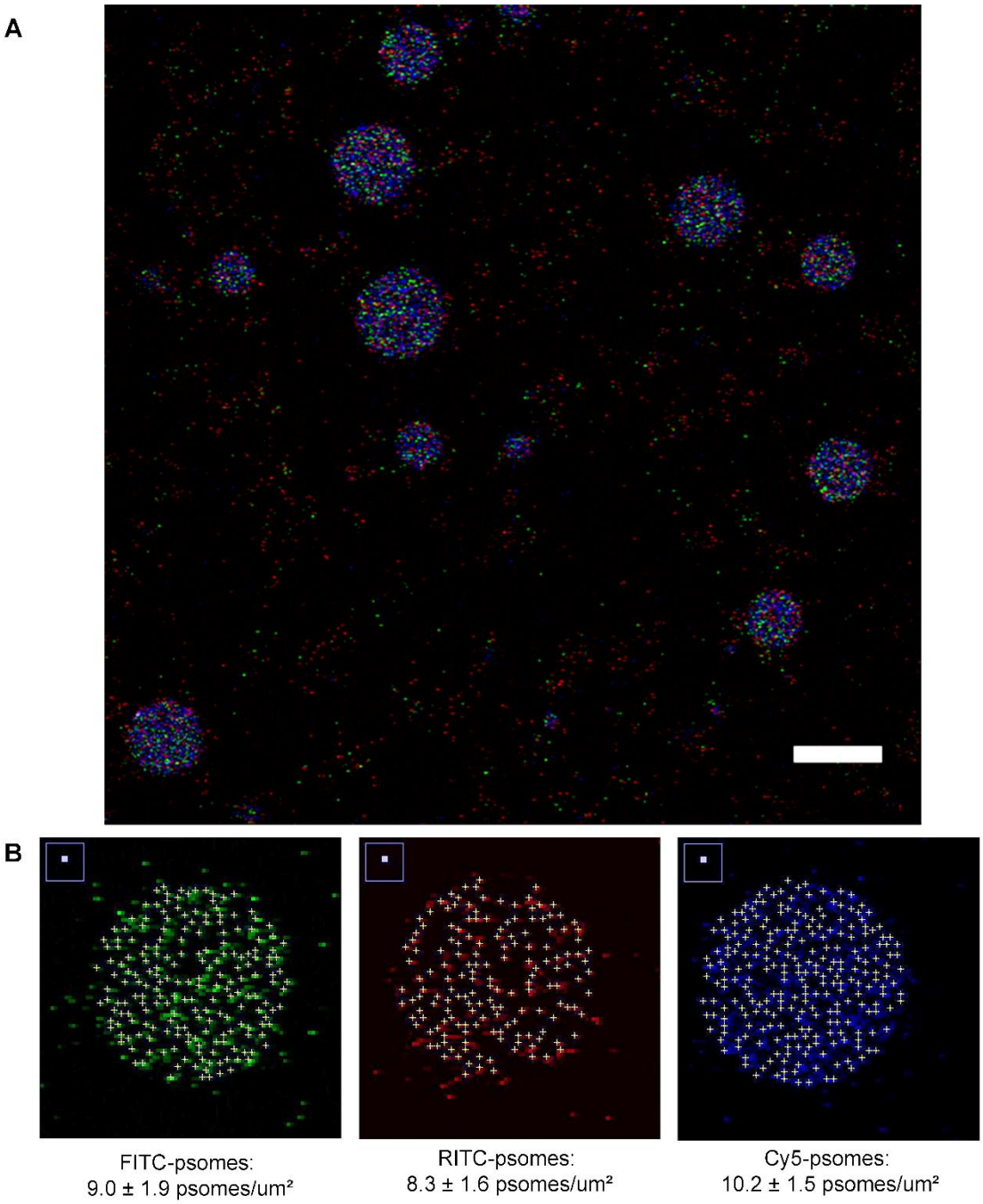


Figure S9: **A.** Wide field confocal image of 3 discrete polymersome populations (seen in Figure 1A), demonstrating equal sequestration of each population (scale bar represents 10 μm). **B.** Individual punctae were counted using ImageJ, then divided by the area of each protocell in which those punctae appeared to obtain an average number of polymersomes per coacervate protocell area. Utilizing an estimated z-resolution of 1 μm, the total density of encapsulated polymersomes is approximately 27 polymersomes /μm³, or for a protocell with 10 μm diameter (V = 523.6 μm³), approximately 14000 polymersomes per protocell.

Figure S10: GOX and HRP reaction progress curve for other glucose concentrations

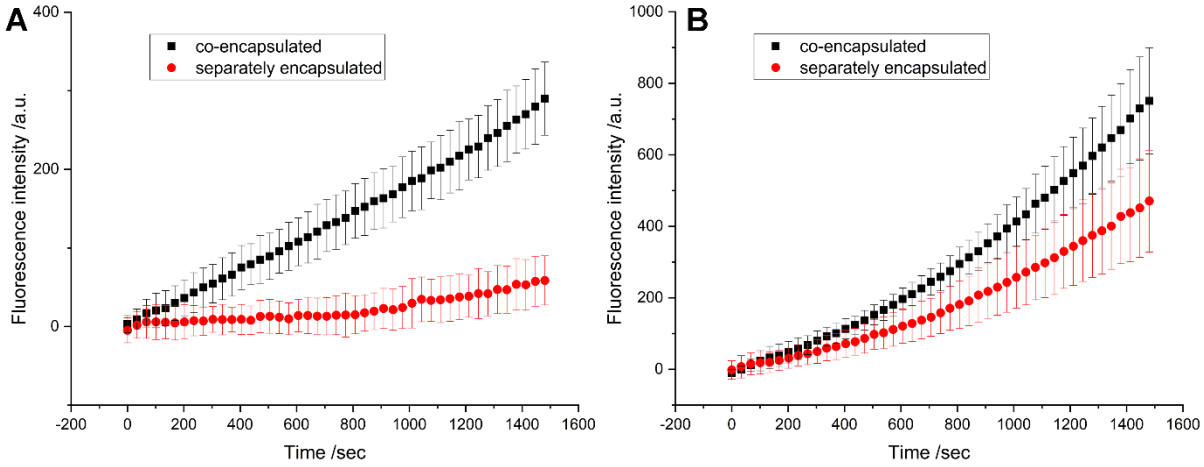


Figure S10: The reaction progress curves for the production of resorufin for protocells given 1 mM D-glucose (**A**) and 5 mM D-glucose (**B**).

Figure S11: Co-culture experiment

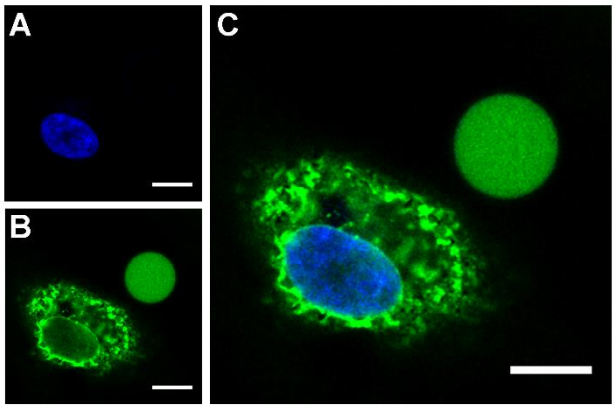


Figure S11: Co-culture experiment where a sub-compartmentalized protocell (FITC-BSA polymersomes) was imaged in the vicinity of a living HeLa cell (nucleus: Hoechst 33342 / membrane: Alexa Fluor 488) to demonstrate both stability and structural homology (scale bar represents 10 µm). (**A**) Hoechst 33342 channel. (**B**) AF488/FITC channel. (**C**) Merged.

3. References

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