Spontaneous membrane-less multi-compartmentalisation via aqueous two-phase separation in complex coacervate micro-droplets

Nicolette G. Moreau,[1] Nicolas Martin,[2] Pierangelo Gobbo,[1] T-Y. Dora Tang[3] & Stephen Mann[1,4 5*]

[1] Centre for Protolife Research and Centre for Organized Matter Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK.
[2] Univ. Bordeaux, CNRS, Centre de Recherche Paul Pascal, UMR5031, 115 Avenue du Dr. Albert Schweitzer, 33600 Pessac, France.
[3] Max Planck Institute of Molecular Cell and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.
[4] BrisSynBio Synthetic Biology Research Centre, Life Sciences Building, University of Bristol, Tyndall Avenue, Bristol, BS8 1TQ, UK.
[5] Max Planck Bristol Centre for Minimal Biology, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK.

*e-mail: s.mann@bristol.ac.uk

SUPPLEMENTARY INFORMATION

1. METHODS

Materials

Polydiallydimethylammonium chloride (PDDA, 8,500 g mol^{-1}) was purchased as a 28 wt% aqueous solution from Polysciences, Inc. Adenosine triphosphate (ATP) disodium salt hydrate, 2,4,6-trinitrophenol-labelled ATP (TNP-ATP) sodium salt, dextran, dextranase, fluorescein isothiocyanate-labelled dextran (FITC-dextran), rhodamine isothiocyanate-labelled dextran (RITC-dextran), fluorescein isothiocyanate (FITC), hydrochloric acid, methanol-d_4, sodium bicarbonate, sodium carbonate, tetraethylene glycol (TEG; $HO[CH_2CH_2O]_3CH_2CH_2OH$) and toluene were purchased from Sigma Aldrich. Sodium hydroxide was purchased from The British Drug Houses (BDH), tetraethylene glycol monoamine (TEG-amine) was purchased from Carbosynth, and 2-[methoxy (polyethyleneoxy)propyl] trimethoxysilane was purchased from ABCR GmbH. RITC-labelled PDDA (RITC-PDDA) and FITC-labelled TEG (FITC-TEG) were synthesized and characterized in-house (see Supplementary Methods). MilliQ water solutions had a resistance of 18.2 MΩ (from a Merck Millipore MilliQ Elix Integral 3 water purification system).

Preparation of coacervate droplet suspension

Stock solutions of PDDA and ATP were prepared at 50 mM in MilliQ water, adjusted to pH 8.0 (± 0.1) with NaOH and stored at 4 °C until use. Coacervate micro-droplets were prepared as a turbid suspension by adding PDDA to ATP at a 1:1 molar charge ratio to reach a final concentration of components of 25 mM. Coacervate suspensions were then centrifuged (10 min, 13,000 × g) to produce a phase separated bulk coacervate phase in equilibrium with a supernatant phase. The supernatant was removed, and the bulk coacervate phase re-suspended in half the volume of water to re-generate coacervate microdroplets with increased stability to coalescence.

Preparation and imaging of TEG-induced vacuolization in coacervate micro-droplets

A 72 µL aliquot of a freshly-made PDDA/ATP coacervate droplet suspension was deposited on a PEG-functionalized imaging dish (see Supplementary Methods). The coacervate droplets were allowed to

settle on the slide for 1-2 min and a region was selected for imaging. Pure TEG, TEG-CH_3 or H_3C-TEG-CH_3 (5-100 µL) was then pipetted into the supernatant solution at approximately 0.5 cm from the imaging region to minimize disruption to the droplets. Additions occurred from the same site in all experiments. Coacervate microdroplets were imaged by bright-field and confocal fluorescence microscopy on a Leica SP5-AOBS or Leica SP5-II confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope equipped with an oil immersion 100× objective lens, 1.4 NA. To observe the localization of the coacervate components, TNP-ATP was doped into ATP to a final concentration of 10 µM and RITC-PDDA was doped into PDDA to a final concentration of 2 mg mL^{-1}. Alternatively, the localization of TEG within the coacervate micro-droplets was determined by doping TEG with FITC-TEG at a FITC-TEG : TEG volume ratio of 1 : 1000. Images were acquired before and after TEG addition, and fluorescence line profiles extracted using *ImageJ* and normalized to the highest fluorescence value. Real-time imaging of the TEG-induced coacervate restructuration was also performed after TEG injection. Analyses of the structures obtained were undertaken on images acquired ~5 min after TEG addition. The number of droplets that contained a single vacuole or no vacuole were counted as a percentage of the total number of droplets in the imaging region. Droplets that contained several small partially formed vacuoles were not included in the counting to avoid overestimating the extent of vacuole formation. The analysis was repeated on three separate samples and the results averaged. Standard deviations were used to plot the error bars. Similar experiments were repeated with tetraethylene glycol monomethyl ether (TEG-CH_3) or tetraethylene glycol dimethyl ether (H_3C-TEG-CH_3) in place of TEG. To determine the number of vacuoles, droplets that had produced only a single vacuole once the system had reached the quasi-equilibrium were counted. These measurements are given as a percentage of the total number of droplets in the population sample. Vacuole size analysis was carried out by measuring the diameter of the vacuole, as a percentage of the diameter of the whole droplet.

TEG/dextran phase separation in coacervate micro-droplets

A 72 µL aliquot of freshly made PDDA/ATP coacervate droplet suspension was deposited on a PEG-functionalized imaging dish. The droplets were allowed to settle on the slide for 1-2 min and a region was selected for imaging. 5 µL of 20 mg mL^{-1} dextran (70 kDa) were added to the coacervate suspension approximately 0.5 cm away from the imaging site, followed by an aliquot of pure TEG. FITC-TEG (doped as 1:1000 (v/v) with untagged TEG) was used to identify the TEG location, and RITC-dextran (70 kDa) was used in place of non-labelled dextran to identify the location of the polysaccharide. Images were acquired before and after TEG addition by bright-field and confocal fluorescence microscopy, and fluorescence line profiles extracted using *ImageJ*. Droplets that produced a single dextran-enriched compartment were measured in the same way as previously described for TEG-only samples. Analyses were performed on three different samples, the results averaged, and the standard deviation reported.

Dextranase-mediated dextran displacement

Freshly made PDDA/ATP coacervate droplets were loaded with dextranase by adding 5 µL of a 2 mg mL^{-1} stock solution of dextranase to 72 µL of a coacervate droplet suspension (final concentration of dextranase 0.13 $mg.mL^{-1}$). This aliquot was deposited on a PEG-functionalized imaging dish and the droplets allowed to settle on the slide for 1-2 min. A region was selected for imaging. 5 µL of 20 mg mL^{-1} FITC-dextran (70 kDa) were added to the coacervate approximately 0.5 cm away from the imaging site, followed by 5 µL of pure TEG (final concentrations of dextran and TEG, 1.2 mg mL^{-1} and 6 wt%, respectively). Alternatively, to identify the localisation of dextranase, non-labelled dextran and FITC-dextranase (at 0.13 mg mL^{-1}) were used. Images were acquired by bright-field and confocal fluorescence microscopy as a function of time. Radial fluorescence profiles on an individual droplet were extracted as a function of time using *ImageJ* across the whole droplet. The boundaries associated with the dextran-enriched inner compartment and droplet/water interface in the fluorescent images were identified from the corresponding brightfield images.

Determination of coacervate water content

1 mL of a freshly made coacervate droplet suspension were supplied with aliquots of pure TEG (equivalent to the microscopy samples), then centrifuged (15 min, 1000 × g) and the supernatant discarded. The remaining coacervate bulk phase was dried in an oven at 100 °C until the dry weight remained constant. The water content of the coacervate phase was calculated using the weight difference before and after drying. Measurements were repeated on three different samples, and the average value and standard deviation reported.

Measurement of ATP partitioning

1 mL of freshly made coacervate droplet suspensions were supplied with aliquots of pure TEG, then centrifuged (15 min, 1000 × g). The resulting supernatant and coacervate phases were diluted by 2 or 200, respectively, in sodium carbonate buffer (200 mM, pH 9) to reduce the amount of scattering from the coacervate microdroplets. The absorption at 280 nm of both fractions was measured on a UV-vis spectrophotometer (PerkinElmer). The absorption values were corrected by taking into account the absorption of TEG at 280 nm. For this purpose, a calibration curve of TEG absorption at 280 nm against TEG concentration was made, and the concentration of TEG in both phases (coacervate and supernatant) was determined from measurements of TEG partition coefficients at each TEG concentration (see Supplementary Methods) by using volumes of supernatant and coacervate phases of 993 µL and 7 µL, respectively. The corrected absorptions at 280 nm were used to calculate the concentration of ATP in both phases, using an extinction coefficient of 15,400 $mol^{-1}.L.cm^{-1}$. Results are reported as the percentage of the mass of ATP in the bulk phase divided by the total mass of ATP (in the bulk and supernatant phases).

Measurements of TEG and FITC-TEG partitioning

10 mL of freshly made coacervate droplet suspensions were supplied with aliquots of pure TEG, then centrifuged (15 min, 1000 × g). The resulting supernatant and coacervate phases were diluted by 2 or 200, respectively, in sodium carbonate buffer (200 mM, pH 9) to reduce the amount of scattering from the coacervate microdroplets. The absorption at 280 nm of both fractions was measured on a UV-vis spectrophotometer (PerkinElmer). Partitioning was determined as the concentration of TEG in the bulk phase divided by that in the supernatant phase.

As the molecular weight of FITC (389.4 $g.mol^{-1}$) is larger than that of TEG (194.2 $g.mol^{-1}$), typical dye-based experiments utilised FITC-TEG at a ratio of 1 : 1000 (v/v) with respect to untagged TEG. Experiments were undertaken to determine whether the larger molecular mass of FITC influenced the partitioning behaviour of TEG. The total amount of TEG and FITC-TEG in the coacervate bulk phase was determined for the following samples that were prepared in pre-weighed vials: (i) PDDA/ATP coacervate + TEG, (ii) TEG, (iii) coacervate + FITC-TEG, (iv) TEG-FITC, (v) coacervate + RITC-dextran (70 kDa) + FITC-TEG and (vi) RITC-dextran (70 kDa) + FITC-TEG. The latter samples were prepared by adding 65 µL of dextran or RITC-dextran to 936 µL of the coacervate droplet suspension, followed by 65 µL of TEG or FITC-TEG (doped 1 : 1000 (v/v) into TEG). In control samples where either TEG or dextran were not included, 65 µL of water was added to maintain equivalent concentrations and volumes between all samples. All samples were centrifuged at 1000 rpm for 10 minutes to gently separate them into bulk coacervate and supernatant. The supernatant was carefully removed by pipetting and the supernatant phase was lyophilised. The resulting powder was re-dissolved in chloroform, in which TEG is soluble but ATP and PDDA are not. The insoluble component was filtered and then chloroform was completely removed from the TEG using a rotary evaporator. The vials were then re-weighed and the mass of TEG calculated. NMR spectra were taken to check that the solubilised product was TEG.

Partitioning of different molecular weight dextrans

Stock solutions of FITC-dextran of varying molecular weight (4,000-2,000,000 g mol^{-1}) were prepared in water at 20 mg mL^{-1}. A 65 µL aliquot of these solutions was added to 935 µL of a freshly-made

PDDA/ATP coacervate droplet suspension (final FITC-dextran concentration of 1.3 mg mL^{-1}), followed by centrifugation (15 min, 1000 ×g). The supernatant and bulk coacervate phases were separated and diluted by 2 or 200, respectively, in sodium carbonate buffer (200 mM, pH 9). UV-vis absorbance was measured in each phase at 492 nm to determine the concentration of FITC-dextran, and the partition coefficient calculated as the bulk coacervate concentration divided by the supernatant concentration.

Construction of the partial TEG/dextran phase diagram

5 µL of dextran (70 kDa) were added to an imaging dish under the optical microscope, followed by the addition of 5 µL of TEG to reach final concentrations for dextran and TEG, respectively, of 2.9 mg mL^{-1} and 86 wt%, 0.5 mg mL^{-1} and 50 wt%, 2 mg mL^{-1} and 50 wt%, 10 mg mL^{-1} and 50 wt%, 1.2 mg mL^{-1} and 6 wt%, and 10 mg mL^{-1} and 25 wt%. A partial phase diagram was determined by observing the presence or absence of phase separation after addition of TEG.

NMR spectroscopy and mass spectrometry

^{1}H and ^{13}C NMR spectroscopy measurements were performed on a 500 MHz Bruker Advance III HD 500 Cryo spectrometer. ^{1}H NMR spectra are reported as δ in units of parts per million (ppm) relative to methanol (δ 3.30, s). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintuplet), dd (doublet of doublets), m (multiplet), and bs (broad signal). Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (*n*) for a given resonance is indicated as *n*H, and is based on spectral integration values. ^{13}C NMR spectra are reported as δ in units of parts per million (ppm) relative to CD_3OD (δ 49.15, septet).
Mass spectrometry was carried out on a Brucker Daltonics micrOTOF II.

Synthesis of FITC-TEG

Tetraethylene glycol monoamine (TEG-amine, 6.6 mg, 34 µmol, 1 eq.) was added to a solution of FITC (13.4 mg, 34.3 µmol, 1 eq.) in deuterated methanol (500 µL). The reaction was left to proceed for 48 hours and the resulting product dried under high vacuum and used without further purification. No free FITC was observed by ^{1}H NMR (at the resolution of the experiment). ^{1}H NMR (500 MHz, methanol-d_4): δ = 8.15 (s, 1H, K), 7.77 (d, *J* = 10, 1H, Q), 7.16 (d, *J* = 10, 1H, R), 6.76 - 6.67 (m, 5H, J, O, P, S, U), 6.56 (d, *J* = 5, 3H, I, M, T), 3.84 – 3.30 (m, 16H, A-H). ^{13}C NMR (150 MHz, methanol-d_4): δ = 191.0 (17), 182.9 (9), 171.4 (16), 163.0, 154.7, 142.4, 142.3, 131.0, 130.8, 130.7, 130.6, 130.5, 130.4, 130.2, 126.3, 126.2, 120.3, 118.9, 114.7, 114.5, 112.0, 103.6, 103.6 (10-29), 73.6 (2), 71.5, 71.4, 71.1, 71.0 (3-6), 67.9 (7), 62.2 (1), 45.5 (8). HRMS (ESI): Calc. for $C_{29}H_{31}N_2O_9S$ [M+1] 583.1745, found 583.1743. Calc. for $C_{29}H_{30}N_2NaO_9S$ [M + Na] 605.1564, found 605.1570.

Synthesis of poly(diallyldimethylammonium chloride)-co-diallylamine-rhodamine (RITC-PDDA)

Diallylamine (26 mmol) was added to a solution of diallyldimethylammonium chloride (DADMAC) (70% w/v) in water. The pH was adjusted to pH 3 using HCl, and 2,2'-azobis(2-methylproionamidine)dihydrochloride (30 mg) was then added. The reaction was left to proceed under nitrogen for 30 mins and then sealed and left under nitrogen for 24 hours at 60 °C. The monomer ratio of diallyldiammonium units to diallylamine was set to 100:1 to maintain normal PDDA functionality within the coacervate system. The PDDA-co-DAA was then filtered to obtain product with molecular weights of 10-50 kDa. 1 mL of RITC (12 mg in 1.3 mL of ethanol) was then added to the PDDA-co-DAA solution. The pH was adjusted to pH 9 using sodium borate buffer solution and the reaction was left to proceed in the dark for 24 hours at room temperature. The product was then dialysed and lyophilised.

Fluorescent labelling of dextranase

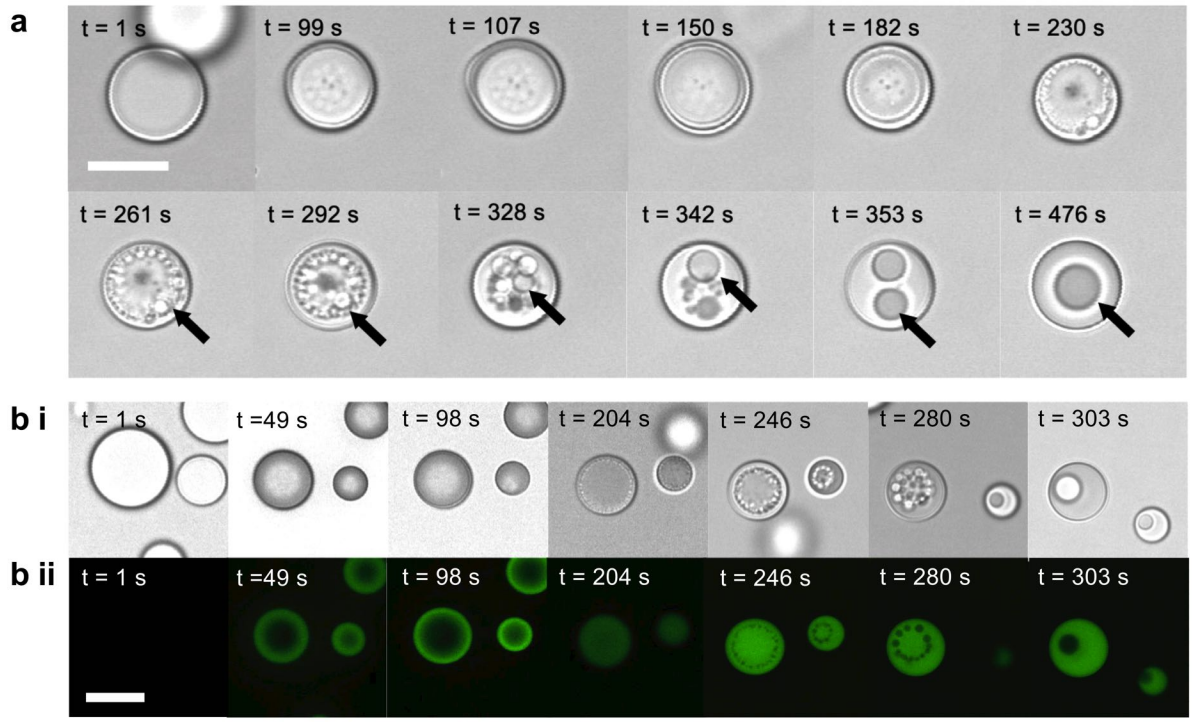
Dextranase was dissolved in 200 mM sodium bicarbonate buffer (pH 9) at a concentration of 6.2 mg mL^{-1}. 140 µL of a freshly-prepared 10 mg mL^{-1} solution of fluorescein isothiocyanate (FITC) in DMSO was added dropwise to 900 µL of the protein solution to reach a FITC:protein molar ratio of ca. 40:1. The reaction mixture was stirred at room temperature in the dark for 4 hours, then purified by size

exclusion chromatography using a Sephadex G-25 resin (Sigma-Aldrich) eluted with Milli-Q water. Fractions were collected and freeze-dried to obtain FITC-labelled dextranase.

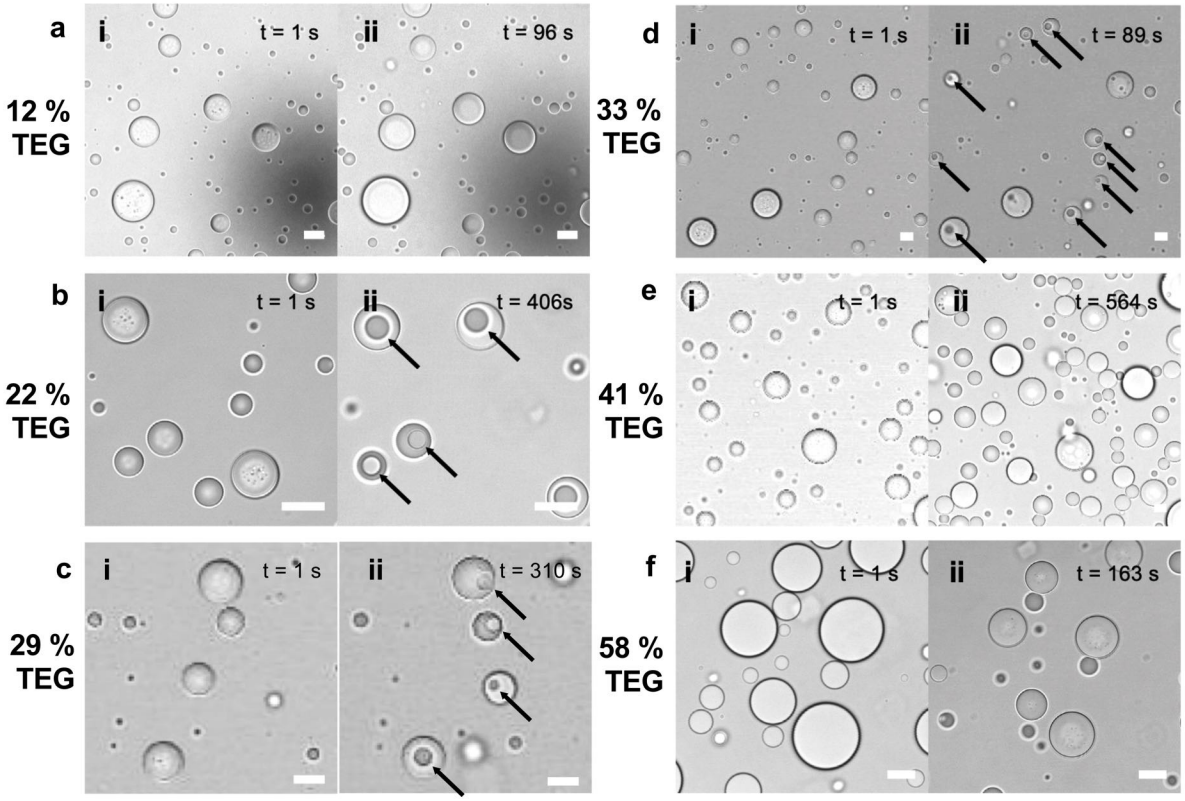
PEG-functionalisation of glass coverslips

Glass coverslips (0.13-0.17 mm thickness) were rinsed with ethanol and water, then dried using a steady stream of nitrogen. The cleaned glass coverslips were functionalised with PEG-by incubation in a 2% (v/v) solution of 3-[methoxy(polyethyleneoxy)propyl]trimethoxysilane dissolved in toluene for 1 hour. The coverslips where subsequently rinsed with water to remove unbound PEG and dried with nitrogen. Functionalised coverslips were glued at the bottom of pierced plastic petri dishes to produce imaging dishes.

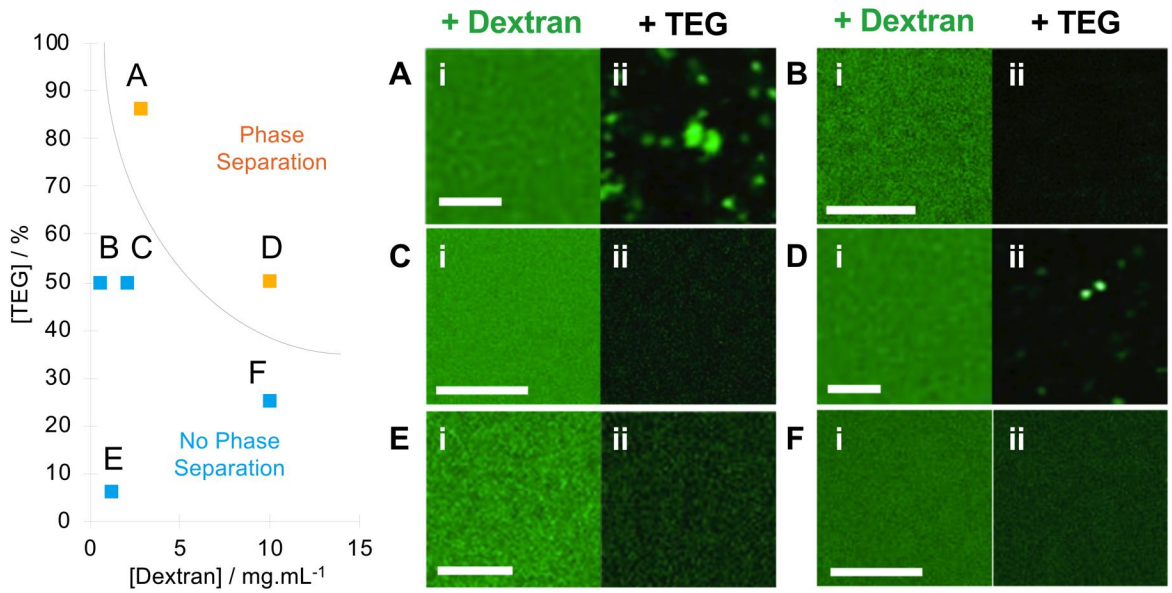
2. Supplementary Figures



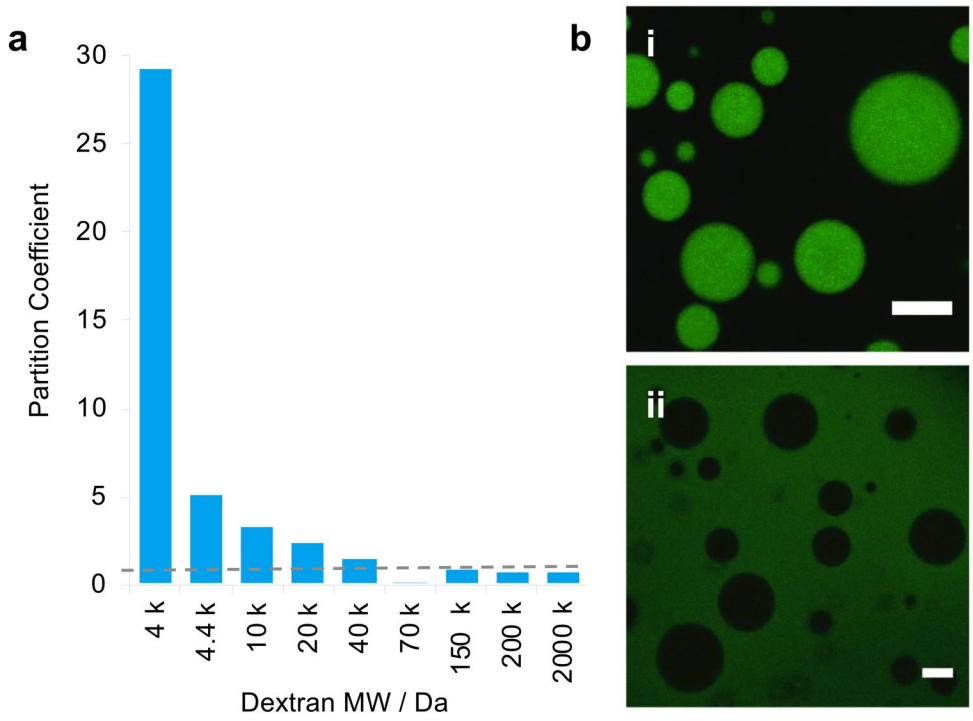
Supplementary Figure 1. TEG-induced vacuole formation in PDDA/ATP coacervate microdroplets. **a**, Brightfield microscopy images of the vacuolization process (black arrows) in a single droplet recorded at different times after *in situ* addition of TEG (22 wt%). Internalized water droplets nucleate within the coacervate, and coalesce into a single large free-moving vacuole. **b**, As in **a** but showing corresponding bright field (i) and green fluorescence (ii) images for several droplets after addition of a mixture of TEG and FITC-TEG. Note the absence/low levels of TEG in the vacuole. Scale bars, 5 µm.



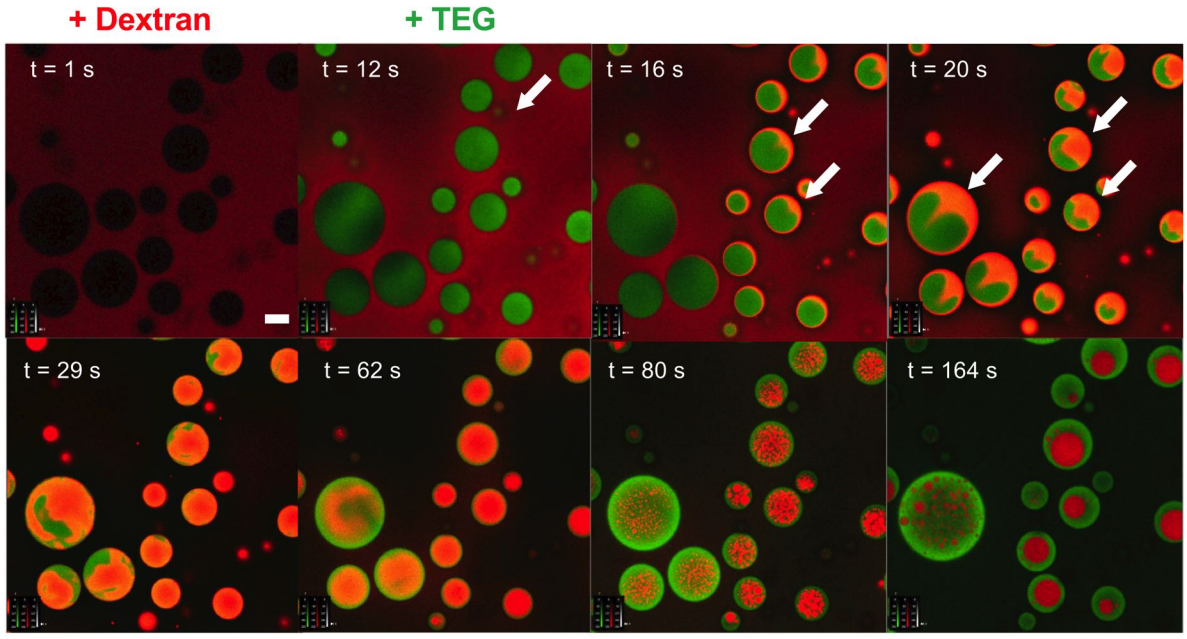
Supplementary Figure 2. Effect of TEG concentration on vacuolization. Brightfield images of droplets (i) before and (ii) after addition of TEG. Vacuoles were not observed for TEG concentrations below 22 wt% (see (a)) or above 33 wt% (see (f)). Arrows indicate single compartments within a coacervate droplet. Scale bars, 10 µm.



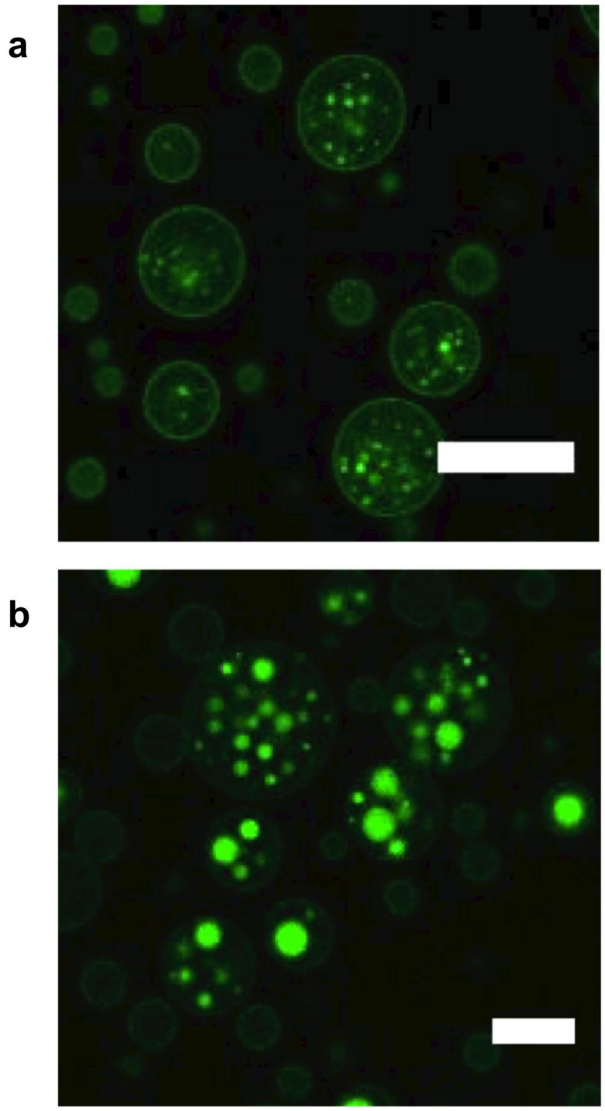
Supplementary Figure 3. TEG and dextran aqueous two-phase separation. Partial phase diagram of TEG and dextran using 150 kDa FITC-dextran (left) and corresponding confocal fluorescence images of FITC-dextran before (i) and after (ii) addition of untagged TEG. Orange and blue data points represent images where liquid-liquid phase separation is present or absent, respectively; grey line shows approximate boundary between one-phase and two-phase systems based on the confocal fluorescence images. Concentrations of FITC-dextran (mg mL^{-1}) and TEG (wt%) respectively are: (A) 3 and 86; (B) 0.5 and 50; (C) 2 and 50; (D) 10 and 50; (E) 1.2 and 6; (F) 10 and 25. Scale bars, **B, C, E, F** = 10 µm; **A, D** = 2 µm.



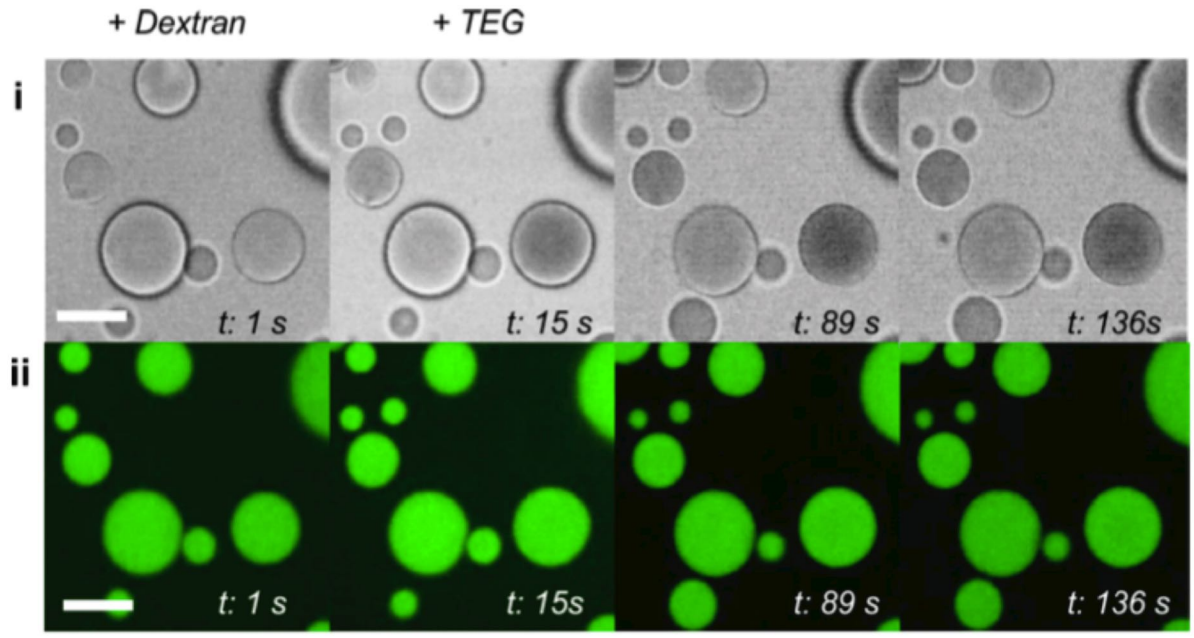
Supplementary Figure 4. Partitioning of dextran into the coacervate droplets is dependent on molecular weight. a, Equilibrium partition coefficients for dextran of increasing molecular weight in PDDA:ATP coacervate droplets. The dotted grey line illustrates the point where the partition coefficient falls below one, indicating that the concentration of dextran excluded from the coacervate is greater than that incorporated. **b**, Confocal fluorescence microscopy images of the coacervates with added FITC-dextran of (i) low (4 kDa) or (ii) high (70 kDa) molecular weight. Scale bars, 10 µm.



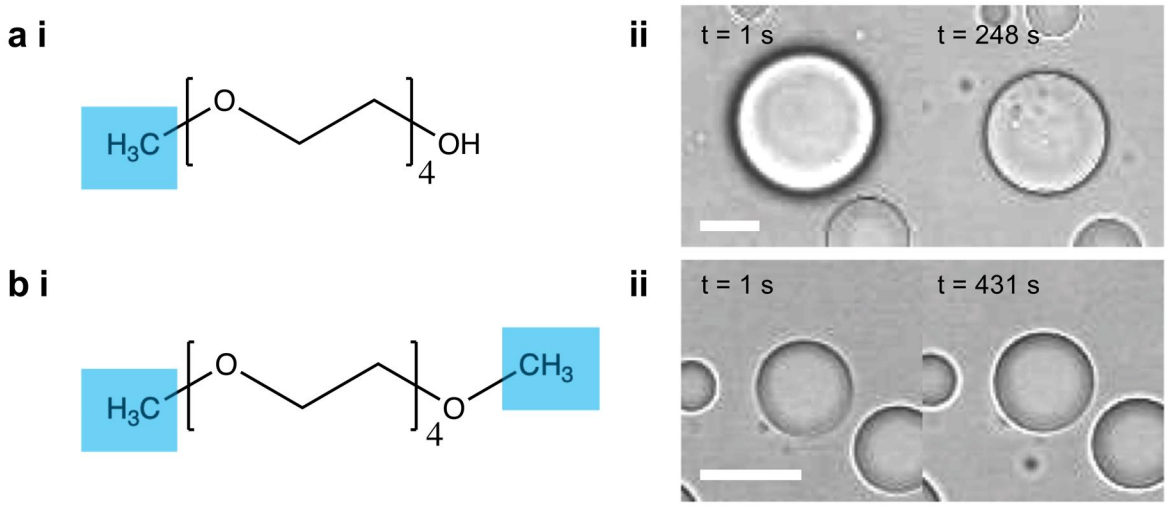
Supplementary Figure 5. TEG-mediated dextran uptake of dextran (150 kDa) and TEG/dextran demixing within PDDA/ATP coacervate micro-droplets. Time series of confocal fluorescence microscopy images of coacervate droplets showing formation of an internal dextran (RITC-dextran)-rich sub-compartment (red fluorescence) after addition of TEG/FITC-TEG. Addition of dextran in the absence of TEG results in exclusion of the polysaccharide (see *t* = 1 s). Subsequent injection of TEG results in sequestration of the oligo-ether seconds (*t* = 12 s), followed by influx of dextran and mixing within the PDDA/ATP coacervate phase (*t* = 16-62 s). Multiple dextran-enriched nuclei are then produced by internalized TEG/dextran demixing (*t* = 80 s) followed by coalescence to produce a single dextran sub-compartment surrounded by a TEG-containing coacervate matrix (*t* = 164 s). The asymmetric uptake of dextran follows the TEG diffusion gradient produced by injection of TEG from one side (right side in the viewed images) of the glass slide. Dextran final concentration 1.2 mg/mL, molecular weight, 70 k; TEG, 6 wt%). Scale bars, 5 µm.



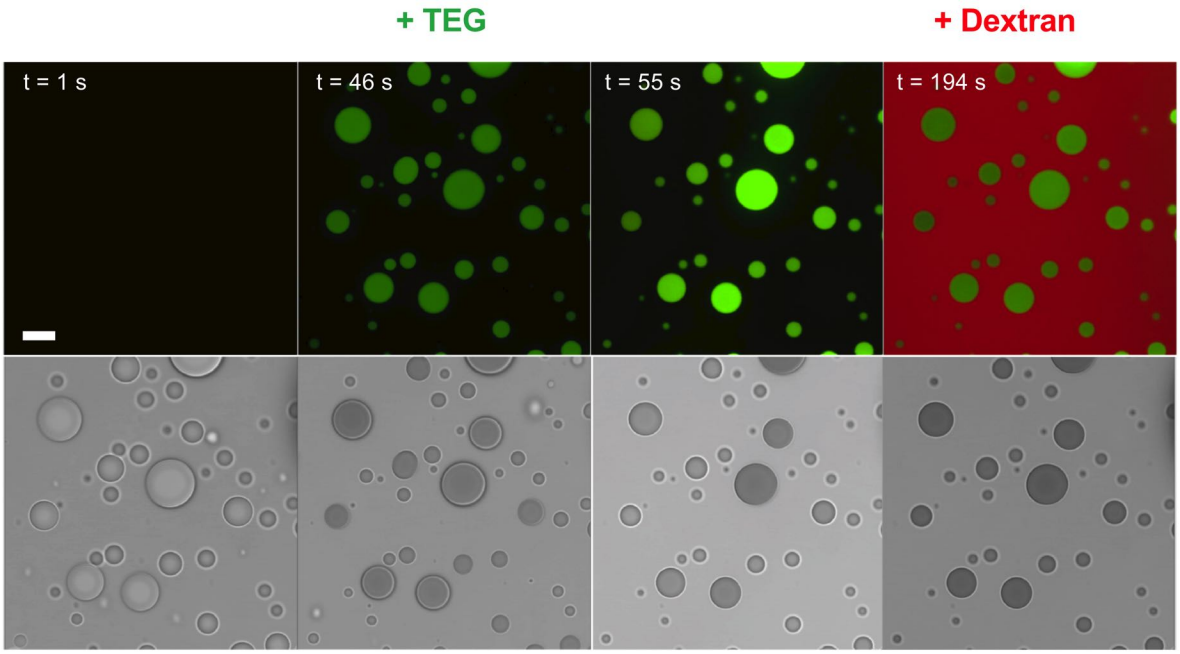
Supplementary Figure 6. TEG/dextran demixing within coacervate micro-droplets at reduced dextran concentrations. Confocal fluorescence microscopy images recorded after TEG/dextran demixing within PDDA/ATP coacervate droplets showing quasi-stable multiple sub-compartments produced at dextran (70 kDa) concentrations of 0.06 (top) and 0.3 (bottom) mg mL^{-1}. Lower dextran concentrations are associated with small phase-separated nuclei. Dextran is labelled with FITC (green fluorescence); TEG, 6 wt%. Scale bars, 10 µm.



Supplementary Figure 7. Absence of TEG-mediated uptake of dextran and TEG/dextran demixing for low molecular dextran (4 kDa). Time series of bright field (i) and corresponding confocal fluorescence (ii) microscopy images of individual coacervate droplets showing sequestration of dextran in the absence of TEG (see image at *t* = 1s). Subsequent addition of TEG did not induce dextran/TEG demixing within the coacervate droplets. Dextran; final concentration 1.2 mg/mL, molecular weight, 4 k. Scale bars, 10 µm.



Supplementary Figure 8. Absence of vacuolization in the presence of end-modified TEG analogues. **a**(i) and **b**(i), Chemical structures of tetraethylene glycol monomethyl (TEG-CH_3) and tetraethylene glycol dimethyl ether (H_3C-TEG-CH_3), respectively. **a**(ii) and **b**(ii), Bright field microscopy images of PDDA/ATP coacervate micro-droplets before and after addition of TEG-CH_3 (22 wt%) or H_3C-TEG-CH_3 (22 wt%), respectively. In both cases, no vacuoles were observed. Scale bars, 10 µm.



Supplementary Figure 9. Absence of TEG/dextran demixing in TEG-loaded PDDA/ATP coacervate droplets. Time series of confocal fluorescence (top row) and bright field (bottom row) microscopy images of individual coacervate droplets after injection of TEG/FITC-TEG showing high levels of uptake (*t* = 1-55 s), followed by addition of RITC-dextran (70 kDa). Dextran is not sequestered when TEG is pre-loaded into the droplets (*t* = 194 s). Scale bar, 10 µm.

3. Supplementary Movies

Movie 1. Confocal fluorescence microscopy video showing vacuole formation induced by the addition of TEG (doped with green fluorescent TEG-FITC) to a PDDA : ATP coacervate droplet suspensions. Corresponding bright field (left) and fluorescence microscopy images are shown for two droplets undergoing reconfiguration to produce multiple internalized water droplets that coalesce into a single vacuole within the TEG-containing coacervate matrix. The images are recorded 219 s after addition of TEG for a duration of 126 s (see Figure S2 for extended time domain). The vacuole formed within the droplet shown on the left disappears from view after 80 s due to ejection fom the droplet into the supernatant. Movie is shown at ~10x speed at 8 frames per second**.** Scale bar, 10 µm.

Movie 2: Bright field (left) and fluorescence (right) confocal microscopy images showing time-dependent changes in the uptake and spatial localization of RITC-dextran (70 kDa) (red fluorescence) after injection of TEG (doped with FITC-TEG (green fluorescence) into a suspension of PDDA/ATP coacervate droplets. Initially, addition of RITC-dextran leads to no uptake of the polysaccharide resulting in fluorescently dark coacervate droplets (see image at t = 87 s). Subsequent addition of TEG/FITC-TEG results in TEG uptake to give green fluorescent droplets surrounded by a red fluorescence background (see image at t = 91s). Within a few seconds, sequestration of dextran into the TEG-loaded droplets occurs (see image at t = 95s; the asymmetric distribution is due to the diffusion gradient incurred by adding TEG to the right hand side of the viewing window). Demixing then occurs at a critical TEG/dextran concentration (see image at t = 147 s) to produce polysaccharide-enriched nuclei that rapidly coalesce to give a single liquid compartment within the TEG-enriched coacervate droplet (see image at t = 252 s). Total duration of recording is 463 seconds in real time. Movie corresponds to Figure 2), and is shown at ~10x speed at 8 frames per second. Scale bar, 10 µm.