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Selective uptake and refolding of globular proteins in coacervate micro-droplets

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Materials

Poly(sodium acrylate) (PAA) and poly(diallyldimethylammonium chloride) (PDDA) with chain lengths of ca. 50 monomers ($5,100 \text{ g mol}^{-1}$ (PAA); $8,500 \text{ g mol}^{-1}$ (PDDA)), bovine serum albumin (BSA), carbonic anhydrase from bovine erythrocytes (CAB) and α -chymotrypsin from bovine pancreas (α -ChT) were used as received. Anhydrous *N*-methylpyrrolidone (NMP), triethylamine (TEA), *N,N'*-dicyclohexylcarbodiimide (DCC), sodium methoxide (25 wt% in methanol), fluorescein amine (isomer II) and fluorescein isothiocyanate (FITC) were used for the fluorescent labelling of polyelectrolytes and proteins. Urea, trizma-base and 1 M solutions of hydrochloric acid and sodium hydroxide were used as buffers, and 3-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (ABCR GmbH) was used to functionalize the surface of microscope capillary slides. Milli-Q water ($18.2 \text{ M}\Omega\cdot\text{cm}^{-1}$) was used for all experiments, and unless otherwise specified, all chemicals and biomolecules were purchased from Sigma-Aldrich.

Fluorescent labelling of proteins

Protein solutions (4 mg mL^{-1}) were prepared by dissolving freeze-dried protein powders in $0.5 \text{ M NaHCO}_3/\text{NaCO}_3$ buffer at pH 9.5. An aliquot of a freshly-prepared anhydrous DMSO solution of fluorescein isothiocyanate (FITC) (10 mg mL^{-1}) was added to the protein solution at a FITC : protein final molar ratio of ca. 20 : 1. The reaction mixture was kept at room temperature in the dark for 2-4 hours, then purified by size exclusion chromatography using a Sephadex G-25 resin (Sigma-Aldrich) eluted with 10 mM Tris-HCl buffer pH 8. The concentration of the FITC-labelled protein in the collected fractions was determined by UV-visible spectrophotometry according to the relationship: $[\text{protein}] = (A_{280} - 0.33 \times A_{495}) / \epsilon_{\text{protein}}$, where A_{280} and A_{495} were the absorbances at 280 and 495 nm, 0.33 the correction factor to account for the dye absorption at 280 nm, and $\epsilon_{\text{protein}}$ the extinction coefficient of the protein ($0.665 \text{ mL mg}^{-1} \text{ cm}^{-1}$ (BSA) and $1.83 \text{ mL mg}^{-1} \text{ cm}^{-1}$ (CAB)). The FITC : protein final molar ratio was determined from the ratio $(A_{495} / \epsilon_{\text{FITC}}) / ([\text{protein}] (\text{mg mL}^{-1}) / M_{\text{protein}})$, where ϵ_{FITC} was the molar extinction coefficient of FITC at 495 nm ($69,000 \text{ mol}^{-1} \text{ L cm}^{-1}$), and M_{protein} the molar mass of the protein ($66,500 \text{ g mol}^{-1}$ (BSA) and $29,000 \text{ g mol}^{-1}$ (CAB)). Typically, the value FITC : protein was ca. 4 : 1.

Fluorescent labelling of PAA

Poly(acrylic acid) was labelled with fluorescein following a published procedure [D. F. Anghel, *et al.*, *Polymer* **1998**, *39*, 3035-3044; E. Spruijt, *et al.*, *Macromolecules* **2010**, *43*, 6476-6484]. Briefly, 0.35 g of PAA (acidic form) was dissolved in 7 mL of *N*-methylpyrrolidone at 60°C . After ca. 1 h, a mixture containing 7.5 μL triethylamine (TEA) (0.02 equiv. compared to acrylic acid monomers) and 11 mg of *N,N'*-dicyclohexylcarbodiimide (DCC; 0.02 equiv.) dissolved in 0.5 mL anhydrous *N*-methylpyrrolidone (NMP) was added to the PAA solution, followed by addition of 19.6 mg of fluorescein amine (0.021 equiv.) in 0.1 mL DMSO. The resulting clear, pale yellow mixture was stirred in the dark at 60°C for 24 h. After cooling to room temperature, PAA was precipitated by dropwise addition of a 25 wt% sodium methoxide in methanol solution (2 equiv.). The orange precipitate was collected by centrifugation (8,000 rpm, 20 min), re-dissolved in a minimum amount of water and precipitated in methanol. This procedure was repeated three times. The final precipitate was dissolved in water and freeze-dried, yielding the sodium salt of fluorescein-labelled PAA as a pale yellow powder. The labelling efficiency was

determined by dissolving a known amount of the labelled polymer in water, adjusting the pH to 9, and recording the UV-visible absorption spectrum. The molar extinction coefficient of fluorescein amine at 490 nm was determined as $76,000 \text{ mol}^{-1} \text{ L cm}^{-1}$ at pH 9. The labelling ratio was 1 : 14800, equivalent on average to the labelling of 1 per 270 polymer chains; thus, the probability of more than one fluorescein label per polymer was low and was not expected to influence the phase behaviour of the labelled-PAA/PDDA mixtures.

Zeta potential measurements

Zeta potential measurements were recorded at room temperature on PDDA/PAA coacervate micro-droplets prepared in 10 mM Tris-HCl (pH 8) at different PDDA : PAA monomer ratios (0.55-1.4) with a fixed final PDDA monomer concentration of 1 mM. Samples were injected into a disposable folded capillary cuvette and analysed using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments). The average value and standard deviation of three successive measurements were determined.

Turbidimetry

Turbidity was used to qualitatively assess the extent of coacervation as a function of urea concentration. Measurements were performed at 500 nm and 25°C using a plate reader equipped with a UV-vis spectrophotometer (CLARIOstar, BMG LabTech).

Determination of coacervate composition, water and urea content, density and refractive index

Stock solutions of PAA and PDDA were prepared at a monomer concentration of 640 mM (60 g L^{-1} (PAA); 103 g L^{-1} (PDDA)) in 10 mM Tris-HCl buffer, pH 8, in the absence or presence of 10 M urea. A PDDA/PAA coacervate was prepared from an equimolar mixture of the oppositely charged polyelectrolytes (5 mL, 80 mM final monomer concentration) by mixing aliquots of the polymer stock solutions in 10 mM Tris-HCl buffer at pH 8 and supplemented with appropriate volumes of a 10 M urea stock solution to give final urea concentrations between 0 and 10 M (with 1M increments). The coacervate suspensions were shaken vigorously and then left to equilibrate for 2 days at room temperature, after which a clear polyelectrolyte-rich phase (coacervate) sedimented. Centrifugation (5,000 rpm, 10 min) ensured complete phase separation between the denser coacervate phase and the polymer-poor supernatant. A given volume of the sedimented coacervate phase was weighed to determine its density, and then dried in an oven at 110°C until the dry weight remained constant. The water content of the coacervate was calculated using the weight difference before and after drying.

To determine the polyelectrolyte composition of the coacervate, 50% of the PAA used for PDDA/PAA coacervation was replaced by FITC-labelled PAA. The concentrations of FITC-PAA in the two phases produced by centrifugation were measured by UV-vis spectrometry ($\lambda_{\text{max}} = 490 \text{ nm}$) after adjustment of the supernatant pH to 9 by dropwise addition of a 1M NaOH solution, or after dilution of an aliquot of the coacervate phase into 0.5 M NaCl at pH 9 to disassemble the PDDA/PAA complex. The AA monomer concentration was determined from both phases using the determined labelling ratio of the FITC to PAA chains (1 : 14800 molar ratio; 1 per 270 polymer chains), the mixing ratio of labelled and unlabelled PAA (1 : 1), and the concentration of the fluorescein dye in both phases.

The refractive indices of the supernatant and the coacervate phase were measured at different urea concentrations using a refractometer (Bellingham & Stanley, London). The values were compared to the refractive indices of pure urea and pure polymer solutions, and the differences used to estimate the distribution of urea between the coacervate and supernatant. The concentrations of urea in the coacervate and supernatant were also estimated from the dry weight of the coacervate phase and the measured content of PAA, assuming a constant PDDA/PAA molar ratio for coacervates prepared in different concentrations of urea. Both methods gave similar estimations of the urea content.

Figure S1. Plots showing urea-dependent change in the equilibrium partition coefficient (K) of FITC-labelled proteins (BSA, CAB), and uncoupled fluorescein.

Figure S2. (a) (i) Confocal fluorescence microscopy images and 3D reconstructions of single CAB-FITC-containing PDDA/PAA coacervate droplets (pH 8; final monomer concentration, 25 mM; monomer molar ratio = 1:1) prepared at urea concentrations between 0 and 10 M showing changes in the spatial distribution of CAB during protein unfolding; scale bars 5 μm. (ii) Confocal fluorescence microscopy images of single α-ChT-FITC-containing PDDA/PAA coacervate droplets prepared at urea concentrations between 0 and 10 M; scale bars 5 μm. (b,c) Fluorescence intensity profiles (red) averaged from >5 single CAB-FITC-containing (b) or α-ChT-FITC-containing (c) PDDA/PAA coacervate droplets, and associated standard deviations (gray) for samples prepared in the absence (top profile) or presence of 2, 4, 6 and 10 M urea (lower profile). Arrow shows increasing urea concentrations. The distance d was normalized to the size of the droplets d_0 .

Figure S3: Plots showing time-dependent exponential decrease of the mean fluorescence intensity (MFI) inside individual coacervate micro-droplets after addition of various urea concentrations.

Figure S4. Far-UV SRCD spectra showing denaturation of BSA in buffers at increasing urea concentrations.

Figure S5. Plot of turbidity measured at 500 nm against urea concentration for a PDPA/PAA coacervate (final [monomer] = 25 mM; PDPA : PAA = 1:1).

Figure S6. (a) Plots showing linear changes in density (black line) and water content (blue line) of PDDA/PAA coacervates (pH 8; final monomer concentration, 80 mM; monomer molar ratio = 1:1) prepared at urea concentrations between 0 and 8.5 M. A progressive decrease from 80 wt% (no urea) to 55 wt% at 8.5 M urea, and corresponding increase in density from 1.07 to 1.18 $\text{g}\cdot\text{cm}^{-3}$ are observed. (b) Plots showing urea-dependent linear increase in refractive index in aqueous urea solutions (control), coacervate bulk phase prepared as in (a), and supernatant. An increase from 1.38 to 1.43 is observed for the coacervate phase. (c) Plot showing approximately constant PAA concentration in PDDA/PAA coacervate bulk phases prepared as in (a) at increasing urea concentration. (d) Linear plots showing effective urea concentration in the coacervate bulk phase and supernatant as a function of the urea concentration used to prepare the samples.

Figure S7. Plot showing decrease in turbidity for coacervates prepared from mixtures of PDPA and BSA ($[BSA] = 0.6 \text{ g L}^{-1} = 9 \text{ } \mu\text{M}$, BSA : PDPA weight ratio = 10:1) with increasing urea concentration. The loss of turbidity is associated with disassembly of the coacervate phase due to unfolding of BSA and disassociation of the polymer/protein complex.

Figure S8. (a) Confocal fluorescence microscopy images of BSA-FITC (i) and CAB-FITC (ii) containing PDPA/PAA coacervate droplets prepared at different PDPA : PAA molar ratios (r) ([PDPA monomer] = 25 mM); (negatively and positively charged droplets, $r \leq 0.83$ and > 0.83 respectively); scale bars 5 μm . (b) Plot of mean fluorescence intensity (MFI) measured at the centre of BSA-FITC (black squares) or CAB-FITC (open squares) containing single coacervate droplets against r . (c) Plot of ζ potential of PDPA/PAA coacervate droplets against r .

Figure S9. Confocal fluorescence microscopy images of negatively charged CAB-FITC-containing PDPA/PAA coacervate droplets (PDPA : PAA molar ratio = 0.71; [PDPA monomer] = 25 mM) prepared at urea concentrations between 0 and 10 M; scale bars 5 μm .