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

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ABSTRACT: Intrinsic differences in the molecular sequestration of folded and unfolded proteins within poly(diallyldimethylammonium) (PDDA)/poly(acrylate) (PAA) coacervate micro-droplets are exploited to establish membrane-free micro-compartments that support protein refolding, facilitate recovery of secondary structure and enzyme activity, and enable the selective uptake and exclusion of folded and unfolded biomolecules, respectively. Native bovine serum albumin, carbonic anhydrase and α-chymotrypsin are preferentially sequestered within positively charged coacervate micro-droplets, and unfolding of these proteins in the presence of increasing amounts of urea results in an exponential decrease in the equilibrium partition constants, as well as the kinetic release of unfolded molecules from the droplets into the surrounding continuous phase. Slow refolding in the presence of positively charged micro-droplets leads to re-sequestration of functional proteins and restoration of enzymatic activity; however, fast refolding results in protein aggregation at the droplet surface. In contrast, slow and fast refolding in the presence of negatively charged PDDA/PAA droplets gives rise to reduced protein aggregation and misfolding by interactions at the droplet surface to give increased levels of protein renaturation. Together, our observations provide new insights into the bottom-up design and construction of self-assembling micro-compartments capable of supporting selective uptake and refolding of globular proteins.

INTRODUCTION

The electrostatic- and entropic-mediated spontaneous liquid-liquid phase separation in water of oppositely charged polyelectrolytes and/or small molecules (complex coacervation) provides a simple pathway to chemically enriched micro-compartments suspended within a component-deficient aqueous continuous phase.1 2 3 Coacervate micro-droplets are currently being investigated as membrane-free artificial cellular platforms due to their propensity to sequester a wide range of biological molecules and machinery, exhibit enhanced enzymatic activity, and support de novo protein synthesis.3 4 5 6 Preferential partitioning within the micro-droplets of watersoluble solutes of various size and composition (molecular dyes, proteins, inorganic nanoparticles etc) despite the absence of a physical membrane is controlled by intrinsic chemical, structural and polarity differences between the molecular crowded coacervate matrix and surrounding continuous water phase.3 7 8 In the case of globular proteins, factors such as molecular size and shape, charge density, and surface charge distribution have been invoked to explain differences and pH-dependent changes in the uptake or exclusion of these biomolecules in the presence of coacervate micro-droplets.3 7 Interestingly, the size, shape, local surface charge density and hydrophobicity of proteins strongly correlate with the folding state of these biomolecules.3 9 10 As a consequence, partly and fully unfolded conformers generally exhibit lower solubility due to exposure of hydrophobic domains and decrease of local charge density, and are usually larger in size due to partial loss of the compact native globular structure.3 9 10 11 Accordingly, the sequestration of protein molecules within coacervate micro-droplets should be dependent on their folding state, and herein we test and develop this hypothesis as a step towards the construction of coacervate-based micro-droplets capable of supporting selective uptake and refolding of globular proteins. Previous studies on the design of artificial chaperones have been inspired by naturally-occurring molecular chaperones,12 13 and based principally on the differential association of proteins in folded or unfolded states with various molecular or nanoscale host systems. For example, Rozema and Gellman14 pioneered the use of surfactant micelles as solubilising agents to sequester partly folded protein conformers, which were subsequently released in a folded state by addition of a competitor guest molecule such as cyclodextrin. Similarly, artificial chaperones have been designed to capture aggregation-prone folding intermediates by non-covalent, reversible complexation with colloidal and interfacial assemblies based on non-ionic nanogels of hydrophobized polysaccharides,15 thermo-responsive polymers,16 17 polymer micelles,18 polymer nanoparticles,19 20 amphiphilic polyelectrolytes21 22 (including those used for integral membrane proteins23), liposomes,24 charged inorganic nanoparticles,25 26 mesoporous materials,27 self-assembled nanotube hydrogels,28 or synthetic or naturally-occurring polyelectrolytes.21 22 29 30 31 32 33 34 35 36 In most cases, recovery of the native-like proteins is achieved by subsequent dissociation of the artificial chaperone/protein complex by changing the buffer, temperature, pH or ionic strength, or via addition of a competitive guest molecule to release the folded protein molecules into the surrounding environment. In contrast, in this paper we exploit intrinsic differences in the molecular sequestration of folded and unfolded proteins within coacervate micro-droplets to establish membrane-free aqueous micro-compartments that support protein refolding, facilitate recovery of secondary structure and enzyme activity, and enable the selective uptake and exclusion of folded and unfolded
molecules, respectively. Specifically, we determine the equi-
librium partitioning of three different proteins, bovine serum
albumin (BSA; 66.5 kDa, pI = 4.5), carbonic anhydrase (CAb;
29 kDa, pI = 5.9) and α-chymotrypsin (α-CT; 25 kDa, pI =
8.75) (2-4 mg mL⁻¹) within positively charged coacervate mi-
cro-droplets prepared in the presence of the denaturant urea,
elucidate the mechanism of urea-dependent uptake and exclu-
sion (or release) of folded and unfolded proteins, respectively,
and investigate the coacervate-mediated refolding and seques-
tration of functional proteins. Our results indicate that transient,
dynamic binding of the partly folded protein conformers to the
coa Servate micro-droplets curtails protein aggregation and
misfolding during the refolding pathway. These observa-
tions provide new insights into the bottom-up rational design
of self-assembling, functional droplets that support the select-
ive uptake and refolding of globular proteins. Given that coa-
Servate micro-droplets have been recently exploited as mem-
brane-free artificial cellular models, our results pave the way
towards the design and construction of synthetic protocells capa-
cable of stabilizing protein structures under adverse con-
ditions.

EXPERIMENTAL SECTION
Partitioning of folded and unfolded proteins in coacerve-
tate micro-droplets
Aliquots of stock solutions of FITC-labelled bovine serum al-
bumin (BSA; 66.5 kDa, pI = 4.5), carbonic anhydrase (CAb;
29 kDa, pI = 5.9) or α-chymotrypsin (α-CT; 25 kDa, pI =
8.75) (2-4 mg mL⁻¹) were added to a 1.5-9.15 mM aqueous
solution of poly(diallyldimethylammonium chloride (PDDA, Mw =
8,500 g mol⁻¹, ca. 50 monomer units) in 10 mM Tris-
HCl at pH 8, and supplemented with the desired urea con-
centration. The mixtures were incubated for 24 hours to ensure
complete equilibrium unfolding of the proteins, and then coac-
ervate formation was induced by addition of poly(sodium acrylate)
(PAA; Mw = 5,100 g mol⁻¹, ca. 50 monomer units) at a final polyelectrolyte concentration between 1-80 mM and
PDDA : PAA monomer molar ratio of 1:1. The mixture was
centrifuged at room temperature for 24 hours, and the coacervate
phase sedimented by centrifugation (5,000 rpm, 15 min).
The equilibrium partition constant for the fluorescently labelled proteins was determined as the ratio of protein concentration in the coacervate phase over that in the supernatant using UV-vis spectroscopy. Confocal micro-
scopy imaging of the BSA- or CAB-containing coacervate micro-
droplets prepared at 25 mM polymer final concentrations and
different urea concentrations was used to assess the degree of
sequestration or exclusion of the folded and unfolded pro-
teins.
Zeta potential measurements
Zeta potentials were recorded at room temper-
ature on PDDA/PAA coacervate micro-droplets (1 mM PDDA
monomer final concentration; PDDA : PAA monomer ratio
= 1 : 1) prepared in 10 mM Tris-HCl (pH 8) and supplemented
with 0-10 M urea. Samples were injected into a disposable
capillary cuvette and analysed using a Malvern Zetasizer
Nano ZS instrument (Malvern Instruments). The average
value and standard deviation of three successive measure-
ments were determined.
Protein refolding and enzyme activity
Stock solutions (25 g L⁻¹) of denatured BSA-FITC or CAB-
FITC were freshly prepared 24 hours prior to the refolding
experiments by dissolving a known amount of the freeze-dried
proteins into 10 mM Tris-HCl pH 8 buffer supplemented with
10 M urea. The stock solutions were added to a dispersion of
PDDA/PAA coacervate droplets (25 mM PDDA; [PDDA]/[PAA] = 0.55-1.4; 10 mM Tris-HCl pH 8; [urea] = 10
M) to give a final protein concentration of 12 g L⁻¹ (180 and
410 μM for BSA-FITC and CAB-FITC, respectively). Refolding
was induced by one-step dilution of the above dispersion into
a suspension of PDDA/PAA coacervate droplets (25 mM
PDDA) in 10 mM Tris-HCl pH 8 ([protein] = 0.6 g L⁻¹),
followed by incubation for 5 h at room temperature, and then
addition of a further aliquot of buffer (final [protein] = 0.03 g
L⁻¹; [urea] = 0.025M, final [PDDA] = 1.25 mM). Alternatively,
an aliquot of the 12 g L⁻¹ protein/urea/coacervate suspension
was diluted with increasing volumes of a dispersion of
PDDA/PAA coacervate droplets in 10 mM Tris-HCl buffer
(pH 8) such that the urea concentration was decreased step-
wise from 10 to 1 M in 1 M intervals. The solutions were in-
cubated for 30 min at each urea concentration. A final 40-fold
dilution was used to reach a final protein concentration of 0.03
g L⁻¹ and a residual urea concentration of 0.025M. Similar
procedures were undertaken for protein refolding in the ab-
sence of any additive or in the presence of PAA or PDDA
alone.

The enzymatic activity of CAB-FITC after refolding in the presence or absence of PDDA/PAA coacervate micro-droplets
was assessed at t = 5h by monitoring the initial rate of hydroly-
ysis of p-nitrophenylacetate (pNPA) at 25°C using a plate
reader equipped with a UV-vis spectrophotometer (CLARIO-
star, BMG LabTech). Typically, 3 μL of a 100 mM pNPA
stock solution in acetonitrile were injected into 300 μL of the
refolded 0.03 g L⁻¹ CAB/coacervate droplet solution in 10 mM
Tris-HCl pH 8, and the mixture placed in a 96-well plate. The
increase in absorbance at 405 nm was monitored for 120 s and
the gradient (S) of the initial variation of absorbance with time
determined. Similar measurements with buffer alone (no pro-
tein) or with freshly solubilized native protein (0.03 g L⁻¹)
gave corresponding values for the background self-hydrolysis
(Sb) and maximal initial enzymatic rate (Spef), respectively. The
% activity recovery was determined from (S/Sb – Sf)/Sb × 100.
Each experiment was repeated three times, and error bars
were calculated as the standard deviation of the data.

For the solubility measurements, the 12 g L⁻¹ urea-unfolded
protein solutions with/without additives were diluted stepwise or
directly into the refolding buffer to reach a final concentration
of 0.6 g L⁻¹. After incubation for 5 hours, the solution were
diluted twice with 0.5 M NaCl to dissociate the coacervate
droplets, then centrifuged (13,200 rpm, 30 min). Solubility
measurements were derived from the spectroscopic determi-
ation of the concentration of CAB (absorbance at 280 nm) in
the supernatant compared to the value obtained for native
CAB at the same final concentration.

Confocal microscopy imaging of coacervate micro-
droplets
Partitioning of folded and unfolded FITC-labelled proteins in
PDDA/PAA coacervate micro-droplets prepared in wa-
ter/urea solutions at a final poly electrolyte concentration of 25
mM and 1 : 1 molar ratio was monitored by confocal micro-
scopy imaging using a Leica SP5-II laser scanning microscope
attached to a Leica DMI 6000 inverted epifluorescence micro-
scope.
The kinetics of secondary structure formation were monitored by SRCD after one-step dilution of a 12 g L\(^{-1}\) protein/coacervate (r=0.63)/urea (10 M) mixture into the refolding buffer (Tris-HCl 10 mM, pH 8) to reach a final protein concentration of 0.6 g L\(^{-1}\).

**RESULTS AND DISCUSSION**

**Partitioning of folded and unfolded proteins in coacervate micro-droplets**

Dispersions of positively charged coacervate micro-droplets (R_0 ~ 3 ± 1.5 μm) were prepared at pH 8 in a range of water/urea mixtures by incubating urea-containing aqueous solutions of poly(diallyldimethylammonium) chloride (PDDA, M_w = 8,500 g.mol\(^{-1}\), ca. 50 monomer units) with fluorescein isothiocyanate (FITC)-labelled BSA, CAB or α-ChT for 24 h, followed by addition of poly(acrylate) (PAA; sodium salt; M_w = 5,100 g.mol\(^{-1}\), ca. 50 monomer units) to give a final PDDA : PAA monomer ratio of 1 : 1. Samples prepared in the absence of urea showed an intense fluorescence associated specifically with the coacervate phase, indicating that the folded proteins were strongly sequestered by the PDDA/PAA micro-droplets. Significantly, increasing the urea concentration up to 10 M resulted in a progressive decrease in fluorescence within the coacervate matrix and an increase in fluorescence intensity within the aqueous continuous phase (supernatant) (Figure 1a).

**Figure 1.** (a) Optical images of centrifuged BSA-FITC-containing PDDA/PAA coacervate dispersions (pH 8; final monomer concentration, 80 mM; monomer molar ratio = 1:1) prepared at urea concentrations between 0 and 10 M. Changes in the relative fluorescence intensities of the coacervate bulk phase (lower layer) and aqueous supernatant (upper layer) indicate a transition from protein sequestration (folded state) to exclusion (unfolded state) as the urea concentration is increased. (b) Plots showing urea-dependent change in the equilibrium partition coefficient (K) of FITC-labelled proteins (BSA, CAB), and uncoupled fluorescein. Values were normalized to the partition coefficient K0 obtained at 0 M urea concentration.

Spectroscopic determination of the corresponding equilibrium partition coefficients (K = [dye]_{bulk coacervate} / [dye]_{supernatant}) gave typical values of 40 or 2 for BSA-FITC, 9 or 0.7 for CAB-FITC, and 65 or 0.8 for α-ChT in the absence or presence of 10 M of urea, respectively (Supporting Information, Figure S1). Moreover, plots of the normalized K values against urea concentration showed an exponential decrease in the amount of sequestered proteins (Figure 1b), with protein concentrations in the coacervate phase decreasing to less than
a tenth of that determined in the absence of urea. Significantly, corresponding plots for the sequestration of fluorescein alone showed only a small decrease in the partitioning of the fluorescent dye as the urea concentration was increased (Figure 1b), suggesting that exclusion of the proteins from the coacervate phase under denaturing conditions was due specifically to unfolding of the biomolecules.

Given these general observations, we undertook a series of confocal fluorescence microscopy studies to determine changes in the spatial distribution of the fluorescently labelled BSA, CAB and α-ChT proteins in individual coacervate droplets that were prepared as described above under a range of denaturation conditions. In each case, we observed liquid droplets with diameters of a few micrometres, indicating that the spontaneous separation of the PDDA/PAA condensate was not significantly perturbed even at very high urea concentrations (10 M).

In the absence of urea, the confocal microscopy images showed individual droplets that exhibited green fluorescence throughout their interior (Figure 2a, and Supporting Information Figure S2a). The observations were consistent with the high equilibrium partition constants determined for sequestration of the native BSA-FITC and CAB-FITC proteins in the bulk coacervate phase (Figure 1b). Line profiles across the droplets showed a higher fluorescence intensity at the surface of the droplets (Figure 2a and Supporting Information Figure S2b), which was attributed at pH 8 to charge complementarity between the negatively charged proteins (pI$_{\text{BSA}}$ = 4.5, pI$_{\text{CAB}}$ = 5.9) and positively charged droplets (zeta potentials ($\zeta$); +17.8 and +9.5 mV (0 M urea) or +8.5 and +1.3 mV (10 M urea) before and after addition of BSA-FITC, respectively). In the presence of increasing amounts of urea, BSA-FITC and CAB-FITC were progressively excluded from the droplet interior to produce micro-compartments that were effectively devoid of protein molecules except at their surface (Figure 2a,b and Supporting Information Figure S2a(i),(b)).

Similar results were obtained for PDDA/PAA droplets containing α-ChT-FITC; however, in this case, the fluorescence images and profiles recorded for the PDDA/PAA droplets were homogeneous and showed no preferential surface localization of the protein in the absence or presence of urea (Supporting Information Figure S2a(ii),(c)), presumably due to the net positive charge of the α-ChT-FITC enzyme (pI = 8.75). The urea-dependent variation in mean fluorescence intensity inside the droplets exhibited an exponential decay (Figure 2c), with characteristic decay constants of 0.26 and 0.59, and half-life urea concentrations of 3.1 and 1.2 M for BSA-FITC and CAB-FITC, respectively. The faster decay associated with exclusion of the unfolded CAB molecules from the droplets was attributed to a lower interaction strength between the enzyme and coacervate matrix, and was consistent with the lower K value of CAB compared with BSA.

**Figure 2.** (a) Confocal fluorescence microscopy images and 3D reconstructions of positively charged BSA-FITC-containing PDDA/PAA single 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 BSA during protein unfolding; scale bars 5 μm. (b) Fluorescence intensity profiles (red) averaged from >5 single BSA-FITC-containing 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 d0. (c) Plot showing exponential decrease in the mean fluorescence intensity (MFI) measured at the centre of single coacervate droplets (d/d0 = 0.5) as a function of urea concentration for BSA-FITC (black squares) and CAB-FITC (open squares). (d) Confocal fluorescence microscopy images of BSA-FITC-loaded PDDA/PAA coacervate microdroplets before (t = 0) and various times after addition of 10 M urea (final [urea] = 5M); scale bar: 5μm. (e) Plot showing time-dependent exponential decrease of the mean fluorescence intensity (MFI) inside individual coacervate microdroplets after addition of urea (arrow).

The above equilibrium experiments were consistent with kinetic studies in which preformed PDDA/PAA droplets containing sequestered native BSA-FITC were mounted onto a glass slide under water, and then exposed to a few microliters of a 10 M urea solution (final [urea] = 5 M). Release of the unfolded protein molecules from individual droplets was confirmed by recording confocal fluorescence serial images and measuring the corresponding changes in mean fluorescence intensity within the centre of the droplets over time (Figure 2d.e). The data revealed a time-dependent exponential decrease in the average fluorescence intensity inside the coacervate droplets, confirming release of the unfolded protein molecules into the surrounding continuous phase. Typically, the denatured molecules were released from individual droplets with a half-life of ca. 22 s under the above conditions. Similar values for the half-life were determined from the exponential release profiles obtained at lower final concentrations of urea (2 or 3.8 M), although the total decrease in fluorescence intensity was lower at reduced denaturant concentrations (Supporting Information, Figure S3).
Protein unfolding in the presence of increasing amounts of urea was confirmed by far-UV synchrotron radiation circular dichroism (SRCD) spectroscopy (Figure 3). The CD spectrum obtained for native (folded) BSA consisted of two minima at 222 and 208 nm that were characteristic of an α-helical secondary structure.\(^\text{51}\) We exploited the high intensity and low background noise associated with SRCD to determine the structure of the native protein in the viscous highly absorbing coacervate bulk phase, and showed that the latter had negligible influence on the two-dimensional conformation of the protein, which consisted of ~64% α-helices, ~1% β-sheets and 13% turns as determined from deconvolution of the spectra with the BeStSel analysis program (Figure 3a).\(^\text{36}\) The peak intensities and mean residual ellipticity were progressively reduced as the urea concentration was increased up to 10 M to give spectra that were characteristic of an unfolded polypeptide (Figure 3b and Supporting Information Figure S4). The transition from folded to unfolded state was observed for both the coacervate bulk phase, supernatant and aqueous solution (control), and in each case the normalized equilibrium unfolding transitions monitored at 222 nm exhibited a two-state unfolding without the accumulation of any intermediate (Figure 3c), in agreement with previous reports on BSA unfolding.\(^\text{38, 39}\) We therefore used a linear extrapolation method to extract the thermodynamic parameters for BSA unfolding in the coacervate dispersion, supernatant and an aqueous control solution (Table 1 and Supporting Information Methods).\(^\text{40}\) The experimental fits yielded values for the free energy of unfolding (\(\Delta \text{G}^{\text{u,}0}\)) and the urea concentration at mid-transition (\(C_{\text{m}}\)) of +43.1 ± 6.7 kJ mol\(^{-1}\) and 5.3 M, respectively, for denaturation in the control solutions. Similar values were obtained in the supernatant solutions. These values were increased respectively to +64.8 ± 22 kJ mol\(^{-1}\) and 5.8 M in the coacervate bulk phase, indicating that the PDDA/PAA matrix stabilized the folded states of BSA. Complexation of negatively charged polyelectrolytes with globular proteins undergoing urea-induced unfolding has been shown to destabilize the structured conformers due to competition between polymer/protein inter-molecular associations and intra-protein interactions.\(^\text{33, 41}\) Thus, the enhanced stability of the folded conformers observed in the coacervate phase was attributed to the crowded environment experienced by the BSA molecules, which presumably disfavours protein unfolding.

Table 1. Thermodynamic parameters for BSA unfolding derived from fitting the experimental data shown in Figure 2b to Equation 2 (Experimental section).

|          | Aqueous control | Supernatant | Coacervate dispersion |
|----------|-----------------|-------------|-----------------------|
| \(m_0\) (L.mol\(^{-1}\)) | -0.011          | -0.027      | -0.072                |
| \(m_C\) (L.mol\(^{-1}\)) | 0.0017          | 0.0038      | 0.0017                |
| \(\Delta \text{G}^{\text{u,}0}\) (kJ.mol\(^{-1}\)) | 43 ± 7          | 41 ± 7      | 64 ± 22               |
| \(m\) (kJ.mol\(^{-2}\).L\(^{-1}\)) | -8 ± 1          | -8 ± 1      | -11 ± 3               |
| \(C_{\text{m}}\) (mol.L\(^{-1}\)) | 5.3             | 5.4         | 5.8                   |

\(\Delta \text{G}^{\text{u,}0}\) is the value for free energy of unfolding, \(m\) is an adjustable parameter that reflects the dependence of the free energy on the denaturant concentration, and \(\Sigma_0^0\) and \(m_0\) (\(\Sigma_{\text{m}}^0\) and \(m_\text{m}\)) are constant parameters used to adjust the experimental baseline variations in the pre- (post-) unfolding regions. \(\Sigma_{\text{m}}^0\) and \(\Sigma_0^0\) were set at 0 and 1, respectively, to decrease the degrees of freedom. The urea concentration at mid-transition (\(C_{\text{m}}\)) was derived from \(\Delta \text{G}^{\text{u,}0}\) and \(m\) using \(C_{\text{m}} = \Delta \text{G}^{\text{u,}0}/|m|\).

Figure 4. Plot of \(\zeta\)-potential against urea concentration for a PDDA/PAA coacervate (final [monomer] = 1 mM; PDDA : PAA = 1:1).
To elucidate the mechanism of protein exclusion from the PDDA/PAA coacervate droplets upon biomolecule unfolding, we investigated the impact of urea on the properties and chemical composition of the coacervate phase. Although urea is known to induce demicellization of ionic and non-ionic surfactants by modulation of hydrogen bond networks, hydrophobic associations, headgroup solvation and counter-ion binding, there are few studies on the influence of urea on the process of complex coacervation. In general, there was only a small decrease in both the turbidity (coacervate yield, Supporting Information Figure S5) and positive zeta potential values for samples prepared at high denaturant concentrations (Figure 4). The positive ζ values were attributed to a slight excess of positive PDDA units in the 1 : 1 coacervate mixture prepared from strong (PDDA) and weak (PAA; 88 and 71% ionization) polyelectrolytes at pH 8 in the absence and presence of 10 M urea, respectively (data not shown). Sequestration of increasing amounts of urea within the coacervate micro-droplets gave rise to a decrease in water content, increases in both the density and refractive index (Supporting Information Figure S6a,b), and a negligible decrease in polymer concentration (Supporting Information Figure S6d). Significantly, the concentration of urea in the coacervate, which was determined from changes in the refractive index and water content, was essentially the same as in the supernatant (Supporting Information Figure S6d), indicating that urea was not preferentially partitioned into the droplets. As a consequence, the reduced solubility of the unfolded protein in the PDDA/PAA micro-droplets was attributed to a weakening of the interaction strength between the denatured biomolecules and coacervate components. One possibility is that electrostatic and entropic interactions between the denatured protein and PDDA/PAA matrix are reduced due to lowering of the local surface charge density of the biomolecules by unfolding of the globular architecture. This was in agreement with other experiments in which we prepared a coacervate from mixtures of PDDA and BSA (no PAA) and observed progressive disassembly of the polymer/protein condensate as the BSA became unfolded at increasing urea concentration (Supporting Information Figure S7). Moreover, the proposed mechanism was consistent with general observations on the uptake of the native proteins in coacervate droplets prepared with a different net surface charge, which indicated that electrostatic interactions were of primary importance. For example, uptake of negatively charged proteins such as BSA-FITC and in particular CAB-FITC was decreased considerably in the presence of negatively charged PDDA/PAA coacervate micro-droplets prepared for example with a PDDA : PAA molar ratio (r) of 0.55 (Supporting Information Figure S8), whereas the converse was observed for droplets of increasing positive surface charge. As a consequence, CAB-FITC was excluded from the highly negatively charged droplets across the whole range of urea concentrations used (Supporting Information Figure S9).

**Figure 5.** (a) Confocal fluorescence microscopy images showing single positively charged PDDA/PAA coacervate droplets (PDDA : PAA = 1 : 1) in the presence of BSA-FITC in the absence of urea (control native protein) (i), in 10 M urea (before refolding) (ii), after one-step 10-fold dilution in buffer (iii), and after 10-fold stepwise dilution in buffer (iv); scale bars, 5 μm. (b) CD spectra of native BSA, unfolded BSA-FITC in 10 M urea, and after refolding procedures. (c) Confocal fluorescence microscopy images of single positively charged PDDA/PAA coacervate droplets (PDDA : PAA = 1 : 1) in the presence of CAB-FITC in the absence of urea (control native enzyme) (i), in 10 M urea (before refolding) (ii), after one-step 10-fold dilution in buffer (iii), and after 10-fold stepwise dilution in buffer (iv); scale bars, 5 μm. (d) CD spectra of native CAB, unfolded CAB-FITC in 10 M urea, and after refolding procedures.

**Coacervate-mediated protein refolding**

We investigated the ability of positively charged PDDA/PAA coacervate micro-droplets (r = 1) to mediate protein refolding from solutions initially containing denatured BSA-FITC or CAB-FITC in 10 M urea, and then subjected to one-step or step-wise dilution into 10 mM Tris-HCl at pH 8 to reach a final [urea] < 1 M. Confocal microscopy images of individual droplets suspended in denatured solutions of BSA-FITC showed strong fluorescence only at the droplet surface (Figure 5a), consistent with data obtained from the experiments on unfolding (Figure 2a,b), and indicative of minimal uptake of the unfolded biomolecules within the coacervate matrix. In contrast, samples subjected to one-step or multi-step dilution consisted of intact droplets that showed green fluorescence throughout their interior (Figure 5a), consistent with uptake of the folded protein molecules. CD spectra confirmed that stepwise dilution of the urea-containing coacervate suspensions gave rise to folded BSA-FITC molecules with a recovery of 50% of the signal intensity compared with a spectrum of the native protein recorded at approximately the same concentration (Figure 5b). However, refolding of BSA-FITC via a one-step dilution procedure produced CD spectra characteristic of kinetically trapped misfolded/aggregated proteins exhibiting poor secondary structure content (Figure 5b). Stepwise dilution also resulted in refolding of denatured CAB and subsequent sequestration of the globular biomolecule within the PDDA/PAA coacervate droplets (Figure 5c). The corresponding CD spectrum of the refolded protein closely resembled that of native CAB (Figure 5d), exhibiting a minimum at
ca. 210 nm, although the peak intensity was slightly lower than that observed for the native enzyme. In contrast, fast protein refolding involving one-step dilution of urea-unfolded CAB resulted in the formation of fluorescent aggregates at the droplet surface, negligible protein uptake, and CD spectra with low signal intensity (Figure 5c,d).

![Figure 6](image)

**Figure 6.** (a) Solubility and (c) recovered enzymatic activity of CAB-FITC (normalized to that of the native protein) after one-step or stepwise refolding in the absence or presence of coacervate micro-droplets at different PDDA : PAA molar ratios ($r = 1.0$, positively charged droplets; $r = 0.63$, negatively charged droplets). (b) Solubility and (d) recovered enzymatic activity of CAB-FITC (normalized to that of the native protein) after one-step refolding in a control aqueous solution (CAB, blue bars), in the presence of PAA or PDDA alone (blue bars), or PDDA/PAA coacervate micro-droplets (blue bars) prepared at various PDDA : PAA molar ratios ($r$); the corresponding surface charges are indicated. Green regions superimposed on the blue bars show the solubility or recovered enzyme activities in the supernatants obtained from the coacervate dispersions.

Solubility measurements, reported as the percentage of CAB proteins in the supernatant of the centrifuged solution after dissociation of the coacervate droplets by addition of salt confirmed that the aggregation observed during the one-step dilution refolding procedure in the presence of positively-charged PDDA/PAA droplets ($r = 1$) was almost fully suppressed during the stepwise dilution procedure (~80% solubility, Figure 6a) was almost fully suppressed during the stepwise dilution procedure (~80% solubility, Figure 6a). The enzymatic activity of refolded CAB-FITC produced in the presence of positively charged PDDA/PAA coacervates ($r = 1$) by stepwise dilution was assessed by monitoring the initial rate of hydrolysis of p-nitrophenylacetate (pNPA) at 25°C. The mean value for the initial rate in the coacervate medium was 53% of that determined for the native enzyme, and slightly higher than the recovered activity determined after refolding CAB-FITC in the absence of the coacervate using a stepwise procedure (47%) (Figure 6c). The results indicated that under these conditions the positively charged PDDA/PAA coacervate matrix was not only compatible with protein solubility, refolding and recovery of the secondary structure, but also served as a molecularly crowded medium for specific uptake of folded molecules with reconstituted enzyme activity. Interestingly, although the enzyme activity associated with fast protein refolding (one-step dilution) was often < 10%, this was considerably increased up to ca. 45% by preparing negatively charged droplets ($r = 0.63$) (Figure 6c), altogether with an increased protein solubility from < 40% up to ca. 80% (Figure 6a); a similar effect was not observed for multi-step dilution (Figure 6c). Significantly, at $r$ values between 0.55 and 0.71, the negatively charged droplets facilitated protein refolding as the initial rate of pNPA hydrolysis was higher than the rate observed for refolded CAB-FITC prepared in the absence of the coacervate (32%) (Figure 6d). Moreover, the enzyme activity of refolded CAB-FITC in the coacervate phase was considerably higher than in the corresponding supernatants produced by centrifugation of the dispersions (Figure 6d), indicating that the presence of the coacervate droplets was required for enhanced enzyme activity in the recovered proteins. The solubility measurements strongly correlated with the regain of activity (Figure 6b,c), indicating that enhanced protein activity resulted from an increased stability of CAB refolding intermediates with regard to aggregation when bound to coacervate droplets. More precisely, we attributed the colloidal stabilization to the presence of excess PAA molecules associated with the complex coacervate, as control experiments involving CAB-FITC refolding in the presence of aqueous solutions of PAA alone also exhibited a considerable increase in the rate of substrate hydrolysis (up to 75%, Figure 6d) as demonstrated previously, whilst controls undertaken in PDDA alone showed inhibited enzyme activity (5%, Figure 5d) due to irreversible protein aggregation arising from electrostatic attraction between the polymer chains and partially or misfolded protein intermediates (Figure 6b).

Conditions giving rise to an increased protein activity in the presence of PDDA/PAA coacervate micro-droplets ($r=0.63$) were used to monitor the kinetics of refolding by SRCD. The secondary structure gradually evolved from a spectrum characteristic of unfolded polypeptide chains, with a deep minimum at ca. 203 nm, towards the spectrum of native CAB, characterized by a minimum at 210 nm (Figure 7a). The evolution of the ellipticity at 210 nm was used to follow the time-dependent regain of a native-like structure, which showed bi-exponential growth behaviour with characteristic times of 20 min and 41 h (Figure 7b). In contrast, half-times of 140 s and 10-12 min have been reported for the formation of the molten globule and the overall folding of CAB, respectively, in the absence of additives. The hours-long refolding was therefore attributed to interactions with the coacervate matrix and was consistent with days-long refolding reported for CAB in the presence of negatively charged polyelectrolytes.
Supporting In-
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[Figure 7. (a) Time-dependent far-UV SRCD spectra of CAB during one-step refolding in the presence of PDDA/PAA coacerve droplets (r = 0.63). (b) Evolution of the molar residue ellipti-
city at 210 nm with time and bi-exponential fit. The inset shows the initial evolution.]

Overall, the above results indicate that tailoring the surface charge of the coacervate droplets can be exploited for mediating protein refolding under the conditions described (Figure 8). Strong electrostatic interactions between positively charged droplets and negatively charged CAB molecules (pI < pH) facilitate the stable deposition of refolded proteins produced by a stepwise (slow) decrease in the concentration of urea. In this case, the enhanced solubility of partly folded CAB conformers at intermediate urea concentrations facilitates refolding, with the consequence that the active enzyme is sequestered preferen-
tially within the droplets (Figure 8 (top)). In contrast, similar interactions under conditions of fast refolding kinetics (one-step dilution) have a deleterious effect on the recovery of enzyme activity, because of the accumulation and high level of aggregation of protein conformers at the droplet/water interface (Figure 8 (middle)). However, interfacial trapping of the misfolded intermediates is circumvented in the presence of negatively charged coacervate micro-droplets, which favour activity recovery during one-step or multi-step dilution-induced refolding pathways (Figure 8 (bottom)). Interestingly, even though the CAB-FITC molecules are excluded from uptake within the negatively charged droplets (Supporting Information Figure S8), the partly folded proteins appear to be adsorbed dynamically at the droplet surface, and this transient association presumably decreases the concentration of aggregation-prone protein conformers in free solution. These observations are consistent with previous reports on the interfacial association and immobilization of heat-unfolded proteins at the surface of charged nanoparticles, as well as with studies on enhanced refolding yields for proteins in the presence of polyelectrolytes of various surface charge.

Figure 8. Schematic illustration of coacervate-mediated protein refolding after stepwise (top, bottom rows) or one-step (middle, bottom rows) dilution of a 10 M urea-equilibrated CAB-FITC containing PDDA/PAA coacervate droplets mixture. The net surface charge of the coacervate micro-droplets is indicated. Green areas delineate regions where fluorescence from CAB-FITC is observed by confocal microscopy imaging. Stepwise dilution of urea-unfolded CAB in the presence of positively charged droplets ensures concomitant protein refolding and sequestration into coacervate micro-droplets (top row); thus, enzyme activity is recovered to the same extent as in the absence of micro-droplets. One-step dilution of urea-unfolded CAB in the presence of positively charged droplets results in protein misfolding and aggregation at the droplet surface (middle row); no enzymatic activity is recovered after urea removal. One-step or stepwise dilution of urea-unfolded CAB in the presence of negatively charged droplets results in transient protein binding and reduced protein aggregation (bottom row); enhanced enzymatic activity is observed compared with refolding in the absence of the coacervate droplets.

CONCLUSION

Coacervate micro-droplets formed by spontaneous self-
assembly in water of oppositely charged PDDA and PAA poly-
mer chains exhibit differential sequestration properties for FITC-labelled proteins depending on their folding state and the surface charge of the droplets. In the absence of urea, neg-
atively charged proteins (BSA-FITC, CAB-FITC, pI > pH) are readily sequestered into positively charged micro-droplets, whilst increases in urea concentration result in the progressive release of unfolded polypeptide chains into the polyelectrolyte-deficient continuous aqueous phase. The selective exclusion of unfolded proteins is attributed to reduced attractive interactions between the coacervate components and unfolded proteins, possibly due to the decrease of local surface charge density and increase in size (excluded volume effect) of the unfolded proteins. Accumulation of CAB-FITC into positively charged coacervate micro-droplets is achieved during protein refolding upon stepwise decrease of the denaturant concentration. Uptake of protein upon refolding is accompa-
nied by the restoration of enzymatic activity, indicating that coacervate micro-compartment can be safe depositories of folded proteins. Increased yields of protein renaturation com-
pared to refolding in buffered solutions are attained under conditions of low sequestration in the presence of negatively charged coacervate droplets, suggesting that low affinity bind-
ing of the partly folded protein conformers to the coacervate micro-droplets inhibits irreversible misfolding and aggrega-
tion. As coacervate micro-droplets have been recently investig-
gated as rudimentary models of protocell organization and function, our observations could provide new opportuni-
ties for developing chemically compartmentalized micro-
ensembles capable of facilitating protein refolding for use in
diverse areas of cell-free synthetic biology, bionanotechnology and protolife research.

ASSOCIATED CONTENT

Supporting Information. Electronic Supplementary Information available: experimental methods; equilibrium partition constants; optical and confocal fluorescence microscopy images; turbidity, zeta potential, density and refractive index measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

CAB: bovine carbonic anhydrase, BSA: bovine serum albumin, α-ChT: α-chymotrypsin, SRCD: synchrotron-radiation circular dichroism

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