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In vitro gene expression within membrane-free coacervate protocells†

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Cell-free gene expression of a fluorescent protein (mCherry) is demonstrated within the molecularly crowded matrix of a polysaccharide/polypeptide coacervate.

The design, engineering and expression of novel gene architectures and circuitry provide a key overarching technology in synthetic biology. While top-down approaches based on the intelligent re-configuration of living organisms represent the main focus of current activity,¹ there is growing interest in bottom-up strategies that seek to generate minimal representations of synthetic cellularity using biologically informed components and processes.^{2,3} As a consequence, advances in protocell engineering are contributing to a resurgence in the use and development of *in vitro* gene expression systems (IVGES),^{4,5} which provide an attractive approach to off-line biological information storage and processing in synthetic cell-free environments. Integration of *in vitro* transcription/translation modules in synthetic protocells has mainly focused on the synthesis of polynucleotides and proteins within phospholipid vesicles.^{6–8} This approach has provided important steps towards the construction of protocells with archetypal properties such as RNA replication,⁹ sequential gene expression,¹⁰ membrane-pore (α -hemolysin) assembly,⁷ gene switching behaviour,¹¹ and extra-vesicular signalling to bacterial cells.^{12,13} Cell-free gene expression systems have also been encapsulated within water-in-oil emulsion droplets,¹⁴ as well as in non-lipid membrane-bound protocells produced by the spontaneous assembly of protein-polymer nanoconjugates (proteinosomes)¹⁵ or amphiphilic silica nanoparticles (colloidosomes),¹⁶ and in molecularly crowded environments such as aqueous dextran/polyethylene glycol (PEG) two-phase water-in-oil emulsion droplets¹⁷ and condensed droplets of a cell lysate.¹⁸

The latter systems are of particular interest as crowding agents can influence gene expression by volume exclusion effects that reduce diffusional rates, increase and decrease association and dissociation binding rates respectively, and provide mechanisms for fine tuning gene circuit dynamics and enhancing system robustness.¹⁹

We recently showed that coacervate micro-droplets prepared by complexation of cationic polyelectrolytes and anionic mono- or polynucleotides can be used as membrane-free, molecularly crowded protocells with a range of biomimetic characteristics.^{20–25} As cell-free gene expression has not been demonstrated in coacervate-based protocells, we embarked on developing appropriate methodologies to establish this platform technology in chemically enriched, molecularly crowded micro-droplets. Herein, we report the use of a carboxymethyl-dextran/polylysine (CM-dextran/PLys) coacervate for the sequestration and retention of a plasmid-containing IVGES, and demonstrate cell-free gene expression and folding of the red fluorescent protein mCherry at pH 8.

In vitro gene expression was undertaken within a polysaccharide/polypeptide coacervate prepared at pH 8 by mixing equimolar aqueous solutions of CM-dextran ($M_w = 10–15$ kDa) and PLys ($M_w = 5–15$ kDa) (final [CM-dextran monomer]:[PLys monomer] molar ratio $\approx 1:0.5$) (ESI,† Methods). The coacervate suspension was centrifuged, the polyelectrolyte-depleted supernatant removed, and a small aliquot (typically 7 μ L) of an ice-cold IVGES containing the pEXP5-NT/mCherry plasmid (IVGES:coacervate volume ratio = 0.75:1) added (Fig. 1a). The mixture was kept on ice, and then raised to a temperature of 18 °C to initiate gene expression under continuous shaking. The resulting IVGES/pEXP5-NT/mCherry-containing coacervate was homogenous and transparent, and showed no evidence of phase separation over a period of at least 24 h. Significantly, the reaction mixture showed a progressive increase in red fluorescence with time consistent with mCherry synthesis and folding within the coacervate medium (Fig. 1b and c).

We used confocal microscopy to monitor the time-dependent increase in mCherry fluorescence in the coacervate phase at 18 °C (ESI,† Methods). To alleviate problems associated with

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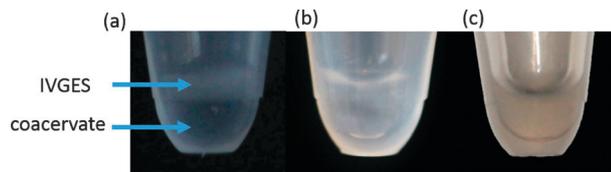


Fig. 1 Optical images showing sample tubes comprising a CM-dextran/PLys coacervate and aqueous solution of a pEXP5-NT/mCherry plasmid-containing IVGES at 18 °C immediately after addition (no mixing) (a), after mixing showing a homogeneous and transparent IVGES/coacervate phase (b), and 24 h after mixing with expression of mCherry in the coacervate (c).

background scattering, aliquots of the coacervate reaction mixture were removed at various time intervals and the extracted polysaccharide/polypeptide matrix disassembled by addition of a high ionic strength buffer containing the antibiotic Kanamycin to terminate protein expression. Each aliquot was centrifuged and the fluorescence spectrum of the supernatant recorded using an excitation wavelength of 594 nm. Emission spectra obtained as a function of time showed a peak maximum at 610 nm that was characteristic of folded mCherry, and which increased progressively in intensity over a period of approximately 12–18 h (Fig. 2a), indicating that transcription, translation and protein folding occurs within the coacervate matrix. The fluorescence intensities were converted to approximate protein concentrations using an appropriate calibration curve, and plots of mCherry concentration against time showed sigmoidal behaviour (Fig. 2b). The data were fitted to a logistic function given by $[mCherry] = K/(1 + \exp(-B(t - t_{1/2})))$ where K is the maximum protein concentration, B the maximum rate in $\mu\text{M min}^{-1}$, and $t_{1/2}$ the time in minutes at the maximum growth rate; *i.e.* the time where half the maximum protein concentration is reached.

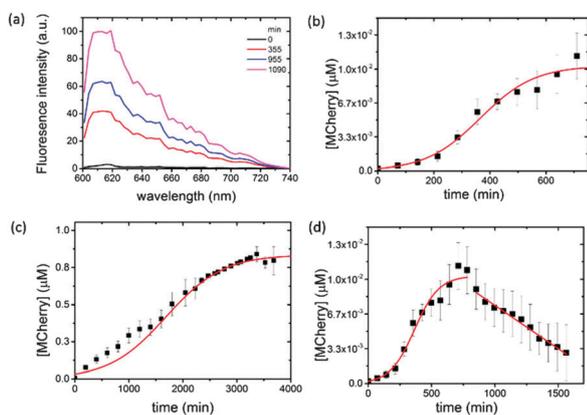


Fig. 2 Plots showing time-dependent changes in (a) fluorescence emission spectra associated with mCherry expression and folding in an IVGES/CM-dextran/PLys coacervate containing the pEXP5-NT/mCherry plasmid at 18 °C after 0 (black), 355 (red), 955 (blue) and 1090 (magenta) min (0, 5.9, 15.0 and 18.1 h, respectively); (b) the concentration of expressed mCherry in the coacervate matrix showing sigmoidal behaviour (red line) over an initial period of 720 min (12.5 h); (c) the concentration of expressed mCherry in aqueous buffer over 4000 min (*ca.* 66 h); and (d) concentration of mCherry in the coacervate matrix over 1500 min (25 h) showing apparent reduction in mCherry concentration due to protein aggregation.

Typically, a maximum protein concentration of $0.0103 \pm 6.03 \times 10^{-4} \mu\text{M}$ was observed at around $t = 720$ min (12.5 h), with a maximum rate of $0.0103 \pm 0.0007 \mu\text{M min}^{-1}$ and $t_{1/2} = 366$ min. A sigmoidal-shaped profile was also observed for the *in vitro* gene expression of mCherry at 18 °C in buffered aqueous solution (Fig. 2c). Interestingly, expression in the control solution was accompanied by an approximately five-fold reduction in the maximum rate ($B = 0.00191 \pm 0.0001 \mu\text{M min}^{-1}$, $t_{1/2} = 1687 \pm 48$ min), which is attributed to an increase in the local concentration of the biological components within the coacervate phase, combined with volume exclusion effects known to be associated with molecularly crowded environments.¹⁹

Although the pEXP5-NT/mCherry plasmid was expressed at a faster maximum rate in the coacervate, this behaviour was not sustained beyond a time frame of approximately 720 min (12.5 h). Fluorescence spectra recorded at later stages (up to 1500 min (25 h)) showed a progressive linear reduction in the intensity at 610 nm due to protein aggregation in the CM-dextran/PLys coacervate (Fig. 2d). As a consequence, the yield was much lower ($K = 0.0103 \times 10^{-4} \mu\text{M}$) compared with the maximum protein concentration obtained in free solution ($K = 0.9 \mu\text{M}$ after 4000 min (*ca.* 66 h)) (Fig. 2c). Indeed, the low yield of expressed mCherry required the use of a combination of antibody trap beads and Western blot analysis to extract, isolate and purify the expressed protein (ESI,† Methods and Fig. S1). Increasing the temperature of the reaction mixture from 18 to 37 °C increased the amount of protein aggregation, although sigmoidal behaviour was still observed over an initial period of 360 min (6 h) (data not shown). As we observed no apparent aggregation of mCherry in the presence of comparable concentrations of aqueous CM-dextran or PLys, we attribute the onset of protein aggregation in the molecularly crowded coacervate phase to a reduction in free volume as the polypeptide chain is continually translated. Occupation of the free space by the folded or unfolded mCherry increases the intermolecular interactions to produce aggregates of the expressed protein. Similar observations have been reported for the *in vitro* and *in vivo* over-expression of proteins within *E. coli* cells²⁶ or artificial crowded environments,²⁷ which lead to the formation of inclusion bodies.

We also undertook control experiments involving the cell-free gene expression of mCherry in aqueous solutions of 100 mM PLys or CM-dextran to assess the influence of the individual polyelectrolytes on protein expression and folding (ESI,† Methods). Fluorescence emission spectra indicated that there was a significant reduction in the yield of mCherry when compared with analogous reactions undertaken in buffered solution in the absence of the cationic polyelectrolyte (Fig. S2a and b, ESI†), suggesting that adverse interactions specifically between components of the IVGES and PLys were also responsible for the low yields, possibly by derailing the transcription/translation apparatus. In contrast, mCherry was successfully expressed and folded in a 100 mM aqueous solution of CM-dextran (Fig. S2b, ESI†).

To study gene expression in individual membrane-free coacervate-based protocells, we used a PDMS microfluidic flow-focusing channel structure to disperse a continuous stream of the pEXP5-NT/mCherry plasmid-containing IVGES/CM-dextran/PLys coacervate into a laminar



- 15 X. Huang, M. Li, D. C. Green, D. S. Williams, A. J. Patil and S. Mann, *Nat. Commun.*, 2013, **4**, 2239, DOI: 10.1038/ncomms3239.
- 16 M. Li, D. C. Green, J. L. R. Anderson, B. P. Binks and S. Mann, *Chem. Sci.*, 2011, **2**, 1739–1745.
- 17 P. Torre, C. D. Keating and S. S. Mansy, *Langmuir*, 2014, **30**, 5695–5699.
- 18 E. Sokolova, E. Spruijt, M. M. K. Hansen, E. Dubuc, J. Groen, V. Chokkalingam, A. Piruska, H. A. Heus and W. T. S. Huck, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 11692–11697.
- 19 C. Tan, S. Saurabh, M. P. Bruchez, R. Schwartz and P. LeDuc, *Nat. Nanotechnol.*, 2013, **8**, 602–608.
- 20 S. Koga, D. S. Williams, A. W. Perriman and S. Mann, *Nat. Chem.*, 2011, **3**, 720–724.
- 21 D. S. Williams, S. Koga, C. R. C. Hak, A. Majrekar, A. J. Patil, A. W. Perriman and S. Mann, *Soft Matter*, 2012, **8**, 6004–6014.
- 22 T. Y. D. Tang, M. Antognozzi, J. A. Vicary, A. W. Perriman and S. Mann, *Soft Matter*, 2013, **9**, 7647–7656.
- 23 J. Crosby, T. Treadwell, M. Hammerton, K. Vasilakis, M. P. Crump, D. S. Williams and S. Mann, *Chem. Commun.*, 2012, **48**, 11832–11834.
- 24 T. D. Tang, C. R. C. Hak, A. J. Thompson, M. K. Kuimova, D. Williams, A. W. Perriman and S. Mann, *Nat. Chem.*, 2014, **6**, 527–533.
- 25 D. S. Williams, A. J. Patil and S. Mann, *Small*, 2014, **10**, 1830–1840.
- 26 D. Hall and A. P. Minton, *Biochim. Biophys. Acta, Proteins Proteomics*, 2003, **1649**, 127–139.
- 27 B. van den Berg, R. J. Ellis and C. M. Dobson, *EMBO J.*, 1999, **18**, 6927–6933.
- 28 D. Van Swaay, T.-Y. D. Tang, S. Mann and A. De Mello, *Angew. Chem.*, DOI: 10.1002/anie.201502886.

