Electronic Supplementary Material (ESI) for Chemical Communications

Stabilization and enhanced reactivity of actinorhodin polyketide synthase minimal complex in polymer/nucleotide coacervate droplets†

John Crosby,* Tom Treadwell, Michelle Hammerton, Konstantinos Vasilakis, Matthew P. Crump, David S. Williams and Stephen Mann*

ESI data

Supplementary Figures and Tables

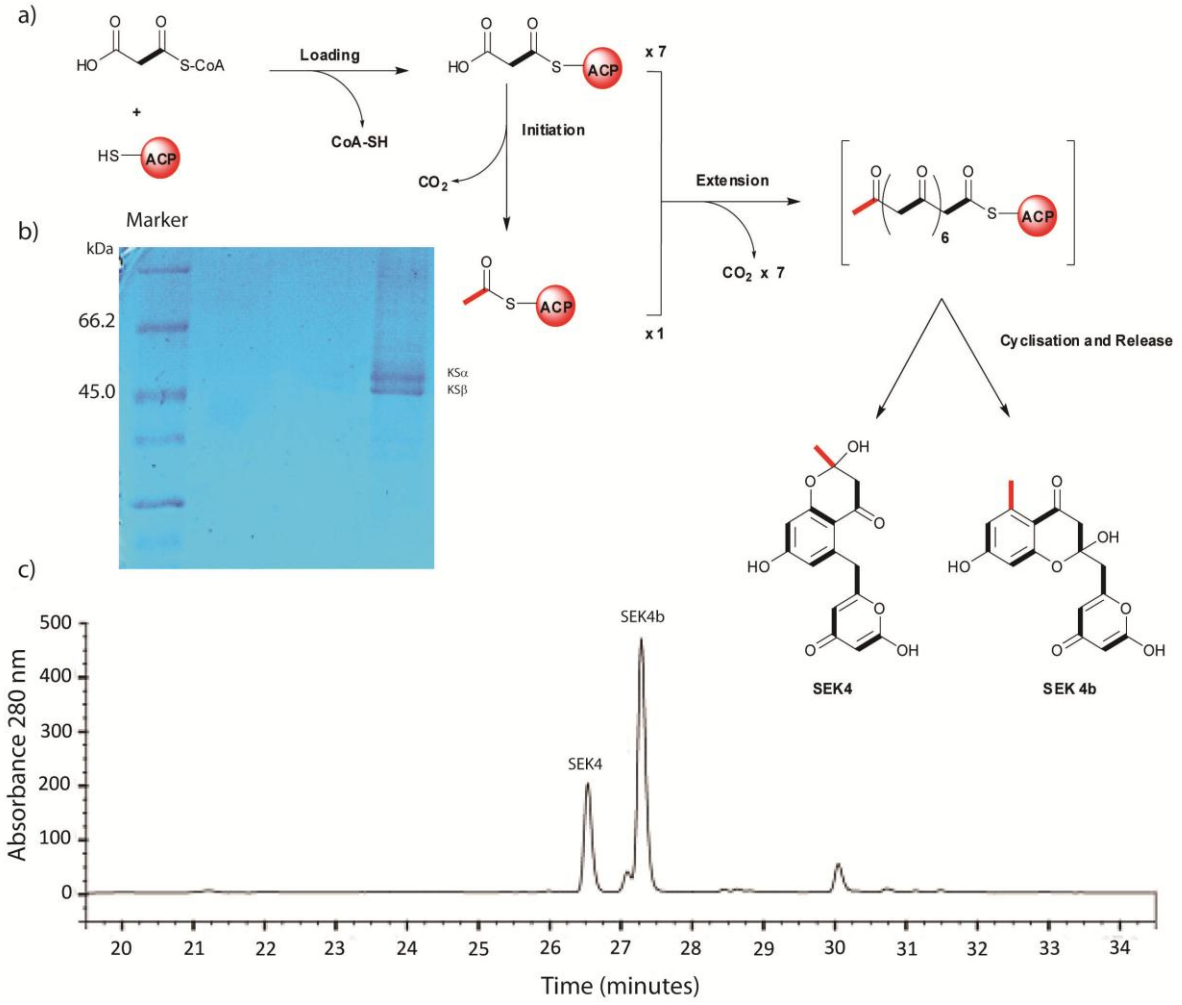

Figure S1 a) Biosynthetic scheme showing the formation of shunt products SEK4 and SEK4b. The former incorporates the correct first ring cyclization while the latter represents an aberrantly cyclized product. b) 10% SDS PAGE analysis showing purification of KS_α/KS_β following nickel chelation and anion exchange chromatography. c) HPLC trace of the extract of the minimal act-PKS assay.

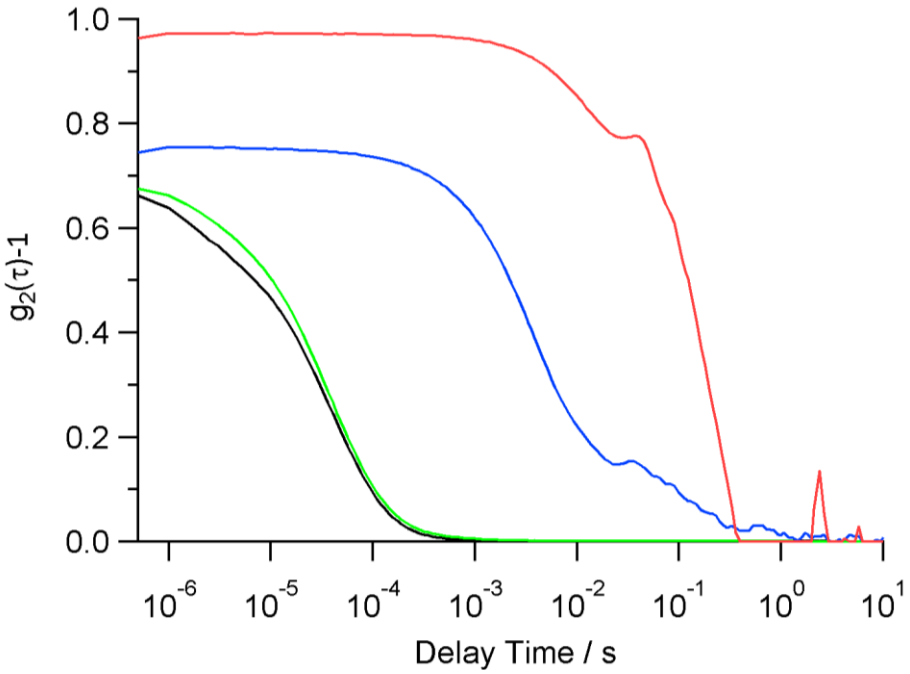

Figure S2 DLS autocorrelation data for coacervate droplets prepared from 2 mM PDDA/ATP at a phosphate buffer concentration of 25 mM after mixing (blue) and after 2 hours (red). In both cases, the data correspond to the presence of stable droplets greater than 10 µm in size. Autocorrelation profiles for samples run in buffer concentrations of 50 (green) and 75 mM (black) are also shown; the curves indicate only the presence of nanoscale clusters (85 and 50 nm, respectively). Relative scattering intensities: 19 x 10^3 (25 mM), 40 x 10^3(25 mM + 2 hrs), 240 (75 mM) and 350 (50 mM). In general, extended periods of micro-droplet stability can be achieved by fine-tuning the PDDA/ATP charge ratio (D. S. Williams, S. Koga, C. R. C. Hak, A. Majrekar, A. J. Patil, A. W. Perriman and S. Mann, *Soft Matter* 2012, **8**, 6004-614). Studies with the minimal PKS assay were not undertaken using the nanoscale clusters: this will be subject of future investigations.

	Supernatant / µM	Coacervate / µM
KS_α/KS_β	0.447 ± 0.00912	7.60 ± 0.228
ACP	2.03 ± 0.0391	296 ± 10.8
malonyl-CoA	4.00 ± 0.115 x 10^4	98.9 ± 0.0527 x 10^4

Table S1. Determined concentrations of minimal PKS proteins and malonyl CoA substrate in coacervate and aqueous supernatant phases.

Materials and Methods

Protein expression: Actinorhodin (act)-polyketide synthase (PKS) *holo*-acyl carrier protein (ACP) was expressed in competent *Escherichia coli* BL21 (DE3) cells transformed with the *act holo*-ACP/pET26b plasmid, and purified by ion exchange chromatography according to previous procedures.[1] Approximately 9 mg of act PKS *holo*-ACP was obtained per litre of cell culture (calculated mass 9,441 Da, observed mass 9,442 Da). Incubation of the purified protein with malonyl-CoA produced an approximately 75% yield of malonyl-ACP (calculated mass 9527 Da, observed mass 9528 Da) within 30 minutes, consistent with self-loading enzymatic activity (Fig. A,B).[2,3] The purified KS_α/KS_β heterodimer was obtained from a cell culture of *Streptomyces coelicolor* (CH999) transformed with the pCB84 KS_α/KS_β expression vector with a yield of *ca.* 32 mg L^{-1}. The high molecular mass complex (*ca*. 90,000 Da; (KS_α = 45,063 Da, KS_β = 42,550 Da)) was purified by nickel chelation and anion exchange chromatography, and analysed by SDS PAGE.

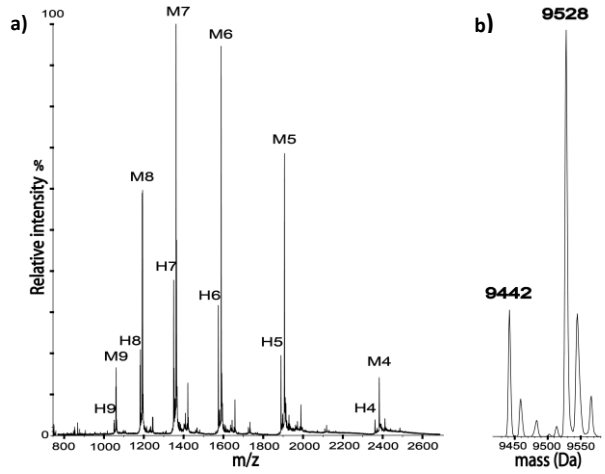

Figure a) Charge envelope of act-PKS *holo*-ACP. Signals labelled H4 to H9 correspond to the unacylated form of the carrier protein, while M4 to M9 indicate the malonylated form. b) deconvoluted ESMS spectrum for act-*holo*-ACP (calculated mass 9442 Da) and malonyl-act-ACP (calculated mass 9527 Da).

Purification of KS_α/KS_β and ACP: *S. coelicolor* act-PKS ACP was purified and analysed using SDS PAGE using standard protocols. The plasmid was transformed into *E. coli* strain BL21(DE3) for isopropyl β-D-thiogalactopyranoside (IPTG)-induced expression.[1,2] Once the $A_{600\ nm}$ of the incubating culture (37 °C) reached a value of 0.6, induction was initiated by the addition of IPTG to a final concentration of 1mM. The C17S mutant of act ACP was used to reduce intermolecular dimerization as well as internal disulfide formation between C17 and the terminal thiol of the 4′-phosphopantetheine (4′-PP) prosthetic group.[3] Purified act ACP was desalted into distilled water using a HiPrep™ Sephadex G-25 HR 26/10 column and lyophilized. Activity of the act ACP was initially followed by self-malonylation.[4,5] For this, act-*holo*-ACP (50 μM) was incubated with malonyl-CoA (50 μM) for 30 minutes at 30 °C in potassium phosphate buffer (100 mM, pH 7.3, 10 % v/v glycerol, 100 μL total assay volume). Reactions were quenched by addition of C4 resin and prepared for mass spectrometric (MS)

analysis using the methods of Winston *et al*.[6] MS analysis was performed using an Applied Biosystems QSTAR® XL hybrid quadrupole time-of-flight instrument. *S. coelicolor* KS_α/KS_β was purified following methods first described by Matharu *et al*.[7] Pooled protein fractions were desalted into 100 mM potassium phosphate buffer (pH 7.3, containing 2 mM DTT, 2 mM EDTA, 10 % v/v glycerol), aliquoted, flash frozen in liquid nitrogen and stored at -80 °C. *In vitro* minimal PKS assays contained act PKS *holo*-ACP, which had been previously incubated with TCEP (2 mM, 60 minutes) to ensure monomerisation of the carrier protein.

Minimal PKS assay: KS_α/KS_β (1 μM) was incubated (10 minutes, 30 °C) with ACP (50 μM) in potassium phosphate buffer (25 mM, pH 7.3) containing glycerol (10% v/v), TCEP (2 mM) and EDTA (2 mM) in a final volume of 100 μL allowing the formation of the active complex. Polyketide production was initiated by the addition of malonyl CoA (1 mM) and assays incubated for a further 2 hours at 30°C with shaking. Following incubation, assays were quenched by addition of solid NaH_2PO_4 (100 mg) and then extracted with ethyl acetate (3 x 300 μL). Following evaporation under nitrogen the residue was dissolved in HPLC grade methanol (100 μL). Extracts were analysed by reverse phase HPLC (Luna 5 μ C18(2) 250 x 4.6 mm column, Phenomenex®) using a Dionex® instrument (P680 pump, ASI-100 automated sample injector and PDA-100 photodiode array detector controlled by CHROMELEON® 6.60 software). The column was equilibrated for 5 minutes post injection with HPLC grade water (Fisher Scientific) containing TFA (0.04 % v/v) at a flow rate of 1 mL/min. The concentration of methanol (HPLC grade, Fisher Scientific) containing TFA (0.05 % v/v) was then linearly increased to 95% over 45 minutes. Compounds were detected by UV absorption at 280 nm. Products SEK4 and SEK4b were quantified using calibration curves of pure standards. For minimal PKS assays incorporating coacervate components, solutions of 50 mM ATP and 50 mM PDDA were added individually and then in combination (50 mM ATP + 50 mM PDDA) to the assay to give final concentrations of both components of 2 mM, maintaining the total volume at 100 μL in each case. The minimal assay was also repeated across a range of salt concentrations (5, 10, 20, 50, 80, 100, 150 and 200 mM NaCl) both in the presence and absence of the coacervate, again keeping the final assay volume at 100 μL.

Partitioning experiments: KS_α/KS_β (1.8 mg/mL, 100 μL,) was added to a solution containing ATP (50 mM, 700 μL) and PDDA (50 mM, 700 μL) and equilibrated for 5 minutes. This solution was then centrifuged (12,480 x g, 20°C, 5 minutes) in order to separate the aqueous and coacervate phases. The concentration of KS_α/KS_β in both phases was determined using the Bradford protein assay, and the partition constant given by the $([KS_\alpha/KS_\beta]_{coacervate}/[KS_\alpha/KS_\beta]_{supernatant})$ concentration ratio. To analyse the coacervate phase, 10 μL was added to 1 M NaCl (190 μL) to disassemble the PDDA/ATP complex. Determination of the ACP partitioning constant was undertaken using essentially the same method with ACP (2 mg/mL, 100μl) replacing the KS_α/KS_β complex.

Malonyl-CoA (50 mM, 10 μL) was added to a solution containing ATP (50 mM, 700 μL) and PDDA (50 mM, 700 μL) and equilibrated for 5 minutes. This solution was then centrifuged (12,480 x g, 20°C, 5 minutes) in order to separate the aqueous and coacervate phases. An aliquot (200 μL) of the supernatant were centrifuged through a molecular weight cut off filter (3 kDa, Millipore, 7,500 x g, 20 °C) until 100 μL of solution was collected. An aliquot of the coacervate phase (10 μL) was added to a NaCl solution (1 M, 190 μL) and treated in the same way as the supernatant. Malonyl-CoA concentrations in both the coacervate and supernatant phases were quantified by HPLC as described above.

Dynamic light scattering studies: Dynamic light scattering (DLS) experiments were performed using a Malvern Zetasizer Nano-ZS equipped with an internal Peltier temperature controller. For coacervate droplet analysis, minimal act PKS assays were reconstituted in the presence of PDDA and ATP with varying concentrations of potassium phosphate buffer (25, 50 and 75 mM, pH 7.3). Light scattering from the solutions was monitored at 30°C and the resulting autocorrelation data fitted using the cumulant method to give the average hydrodynamic diameter along with the derived count rate of the scattering intensity (as given by the Malvern Zetasizer software). Measurements on the coacervate droplets in 25 mM phosphate buffer were also performed after 2 hours, which was equivalent to the incubation length used for the assay. Zeta-potential measurements were also performed on the droplets in 25 mM phosphate buffer.

Bradford protein assay: Protein concentrations were assayed using the Bradford reagent assay (Bradford reagent: 50 mg Coomassie blue G-250 dissolved in ethanol (95 %, 5 mL) and phosphoric acid (85 %, 10 mL) and made up to 1 L with water, filtering to remove undissolved dye).[8] The protein solution (20 μL) was added to the Bradford reagent (1 mL) before UV (595 nm) analysis. A standard curve was prepared using bovine serum albumin (BSA) from 25 to 2000 μg/mL.

1. J. Crosby, D. H. Sherman, M. J. Bibb, W. P. Revill, D. A. Hopwood and T. J. Simpson, *Biochim. Biophys. Acta-Protein Struct. Molec. Enzym.*, 1995, **1251**, 32-42.
2. R. J. Cox, T. S. Hitchman, K. J. Byrom, I. S. C. Findlow, J. A. Tanner, J. Crosby and T. J. Simpson, *FEBS Lett.*, 1997, **405**, 267-272.
3. J. Crosby, K. J. Byrom, T. S. Hitchman, R. J. Cox, M. P. Crump, I. S. C. Findlow, M. J. Bibb and T. J. Simpson, *FEBS Lett.*, 1998, **433**, 132-138.
4. T. S. Hitchman, J. Crosby, K. J. Byrom, R. J. Cox and T. J. Simpson, *Chem. Biol.*, 1998, **5**, 35-47.
5. A. E. Szafranska, T. S. Hitchman, R. J. Cox, J. Crosby and T. J. Simpson, *Biochemistry*, 2002, **41**, 1421-1427.
6. R. L. Winston and M. C. Fitzgerald, *Anal. Biochem.*, 1998, **262**, 83-85.
7. A. L. Matharu, R. J. Cox, J. Crosby, K. J. Byrom and T. J. Simpson, *Chem. Biol.*, 1998, **5**, 699-711.
8. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.