Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary information

for

Atomic Force Microscopy captures folded ribosome bound nascent chains

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Materials and Methods

DNA Cloning

DNA encoding human ankyrin-R repeats 1-24 of the membrane binding domain with residues 1 to 86 of the spectrin binding domain (a kind gift from Van Bennett) was cloned into the poly(I27) pRSET A, vector which was originally developed for single molecule force spectroscopy measurements (a kind gift from Jane Clarke), using BssHII and SpeI restriction sites. The SecM$^{150–166}$ stalling sequence (FSTPVWISQAQGIRAGP) gene was inserted into the same vector using MluI and EcoRI restriction sites. This short sequence (SecM) comprising 17 amino acids (aa) of the 170-aa SecM leader peptide is sufficient to induce translational arrest. The stalled complex has the peptidyl-tRNA (SecM-tRNAGly) at the P-site and Pro-tRNAPro at the A-site of the ribosome, and is thus "frozen" in a pre-translocation state prior to peptide bond formation. When the stall sequence is expressed at the end of nascent chains, stable translation-arrested ribosome complexes accumulate in intact cells or cell-free extracts. The DNA sequence of SecM was adapted from Evans et al. and Iizuka et al. Two oligonucleotides (sequence of SecM flanked with parts of restriction sites necessary for "sticky ends" formation):

5'- cgcgtTTCAGCACGCCCGTCTGGATAAGCCAGGCGCAAGGCATCCGTGCTGGCCCTg – 3'
5' - aattcAGGGCCAGCACGGATGCCTTGCGCCTGGCTTATCCAGACGGGCGTGCTGAAa – 3'
were purchased from IDT and annealed.

The engineered plasmids were transformed into High Efficiency Turbo Competent *E. coli* (NEB, catalog #C2984I) bacterial cells and ethanol precipitated using the QIAprep Spin MiniPrep Kit (QIAGEN, catalog #27104). The $I27_2$–AnkR-I27-SecM construct was cloned from the pRSET A vector to the pRIBA 102 vector (IBA, catalog # 2-3691-000) for expression and efficient purification *in vitro*. The pRIBA 102 vector carries a N-terminal *Strep*-tag which must be used instead of *His*-tag because all proteins purified for the PURExpress® system also contain *His*-tags[29]. Previous studies on effective purification of RNCs[19] have pointed out *Strep*-tag as an effective alternative.

Expression and purification of RNCs

RNCs were obtained with the engineered plasmid expressed using PURExpress® In Vitro Protein Synthesis Kit (NEB, catalog #E6800S) – a cell-free protein synthesis system based on the *E. coli* translation apparatus. The reaction mixture is reconstituted with purified ribosomes, initiation factors, release factors, elongation factors, tRNAs, amino acids, aminoacyl-tRNA synthetases, and several other enzymes required for the translation[29] of the gene encoding the desired protein. The system does not contain chaperones or a trigger factor and is free from proteases. Samples were prepared according

to the manufacturer's instructions and were supplemented with Murine RNase inhibitor (NEB catalog #M0314S). A control sample was prepared by substituting required volume of plasmid with RNase free water to keep the concentrations constant and to prevent expression. After 3 hours of incubation in 37°C, the reaction was stopped by placing the samples on ice. The presence of the desired nascent chains was verified with SDS-PAGE by measuring the molecular mass of the denatured proteins. The resulting bands present, ~141 kDa and ~132 kDa, correspond with the estimated molecular weight of the expression products: $I27_2$AnkR$I27_2$ and $I27_2$–AnkR-I27-SecM$^{150–166}$, respectively (Supplementary Figure 1b). Present in all three samples (control, $I27_2$–AnkR-I27-SecM$^{150–166}$, $I27_2$–AnkR-$I27_2$), the pattern of bands below the molecular weight of ~100 kDa is characteristic for ribosomal proteins. Separation of the RNC fraction was performed using *Strep*-Tactin Spin Columns (IBA, catalog #2-1850-010) according to the manufacturer's procedure with the exception of a longer washing step using buffer R (50 mM Tris-HCl (pH 7.5), 25 mM MgCl2, 150 mM KCl). When the absorption at 260 nm of the last wash fraction exceeded a value of 0.06, additional washing steps were performed. RNCs were than eluted in a total of 3 fractions (100µl each) with buffer R containing 2.5 mM desthiobiotin.

Expression and purification of released nascent chains

Plasmid containing $I27_2$–AnkR-I27-SecM$^{150–166}$ sequence was transformed into *E. coli* OverExpress C41(DE3) pLysS cells (Lucigen, catalog #60442-1). A freshly-grown bacterial colony was inoculated in 6ml LB Broth medium (BD) with 1mM ampicillin at 37˚C overnight. For pre-culture, 1 ml of overnight culture was inoculated into 100 ml LB until OD600 > 0.3 at 37˚C. Expression was induced with 0.5 mM IPTG. The cells were harvested by spinning down at 4.1 k x g for 20 min and then frozen at -80˚C. Thawed cells were resuspended with Lysis buffer including lysozyme and benzonase nuclease (Qproteome™ Bacterial Protein Preparation Kit, QIAGEN, catalog #37900). The lysates were spun down at 14 k x g for 30 min at 4˚C. Strep-tagged AnkR3I27 protein was purified by Strep-tag spin columns analogically to RNCs sample.

AFM Imaging

AFM imaging was performed with a Nanoscope V MultiModeTM Scanning Probe Microscope (Veeco Instruments Inc., Santa Barbara, CA) using the Tapping Mode in the air. The 10 µl protein solution was incubated on a freshly-cleaved mica surfaces (10 mm diameter) at room temperature for 30 sec followed by washing with 1ml of RNase free water and air-drying before experiments. RTESP probes (Bruker AFM Probes, catalog # MPP-11100-10) with resonance frequencies of ~300 kHz were used for air imaging. All images were collected at a scan rate of 1.0 Hz, with 1024x1024 pixels and scan sizes ranging from 1 to 3 µm2. AFM images were analyzed using NanoScope Analysis software Version 1.30 (Bruker). Protein structure representations were prepared in a molecular graphics program VMD30 based on PDB-entry 1TIT for I27 structure, 1N11 for 12 ANK repeat stack from human ankyrin-R structure, 2AVY and 2AW4 for ribosome structure.

Supplementary Figures

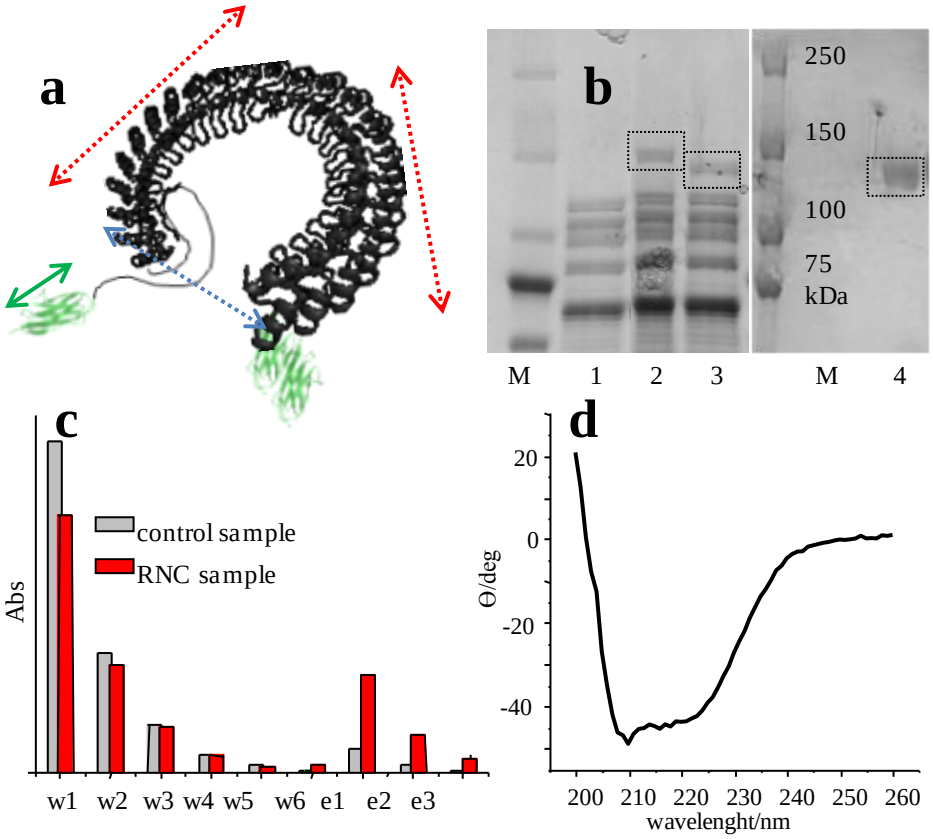

Figure S1 *Isolation of RNCs.* (a) Extrapolated structure of the $I27_2$–AnkR-I27-SecM, construct used to visualize RNCs, created based on available crystallographic data. Arrows showing dimensions of ankyrin-R (red marks the length of each shoulder and blue marks the end-to-end distance =12nm) and I27 (green marks the length = 3.4nm). (b) Denaturing SDS-PAGE of samples obtained through *in vitro* expression (1- control: no DNA in reaction mixture; 2- addition of engineered plasmid with additional I27 module in place of SecM; 3- obtained using plasmid containing SecM; 4- after *in vivo* expression.) (c) Characteristic for ribosomes absorbance at 260nm, measured for each fraction after column purification of sample expressed *in vitro* (w1-w6: washing steps; e1-e3 elution steps). Sample e1 was used for AFM imaging. (d) CD spectrum of $I27_2$AnkR4$I27_2$ with minima at 208 and 220nm, revealing characteristic for this protein dominant α-helical structure.

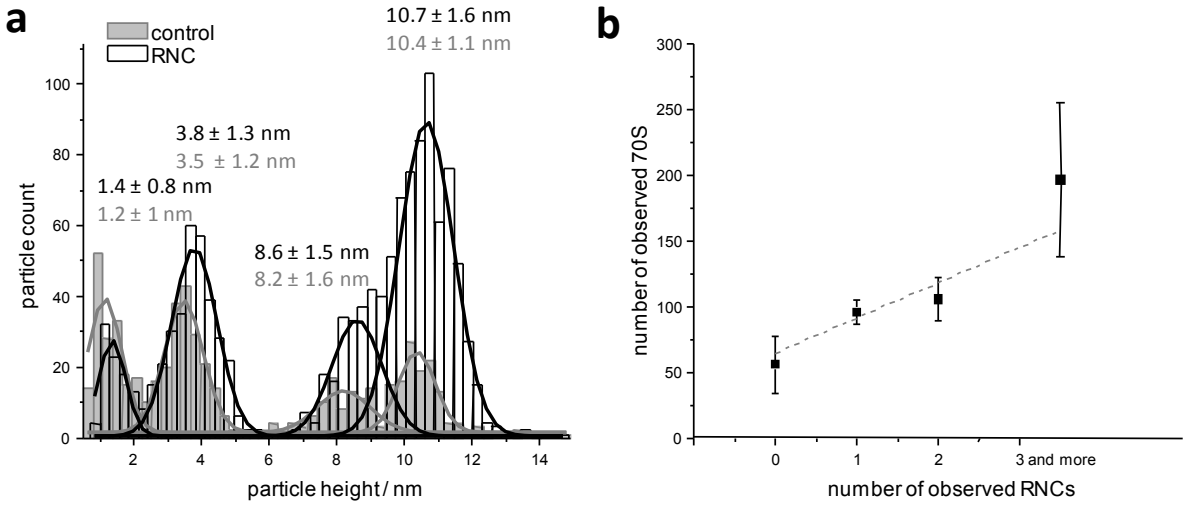

Figure S2 *Characterization of particles by means of height* (a) Representative distribution of particles on two 3μm^2 surfaces covered with control sample (gray) and RNC sample (black) reveals presence of four distributions of particles with heights centered at around 11 nm (RNC sample: 10.7±1.6nm, mean ± s.d., number of particles *n=649, 51.1% of all particles on the surface*; control sample: 10.4 ± 1.1nm, *n=97, 16.6%*), 8.5 nm (RNC sample 8.6±1.5nm; *n=191, 15%*; control sample: 8.2 ± 1.6nm; *n=104, 17.8%*), 4 nm (RNC sample 3.8 ± 1.3nm, *n=313, 24.6%*; control sample: 3.5 ± 1.2nm, *n=197, 33.7%*), and 2 nm(RNC sample 1.4 ± 0.8nm, *n=90, 7%*; control sample: 1.2 ± 1nm, *n=159, 27.2%*). For each image the total number of different particles was counted based on mean height ±s.d. of obtained distribution. Each of these values was normalized based on the total amount of particles observed on each surface. (b) Amount of observed ribosome-bound nascent chains as a function of total number of ribosomes observed on a surface.

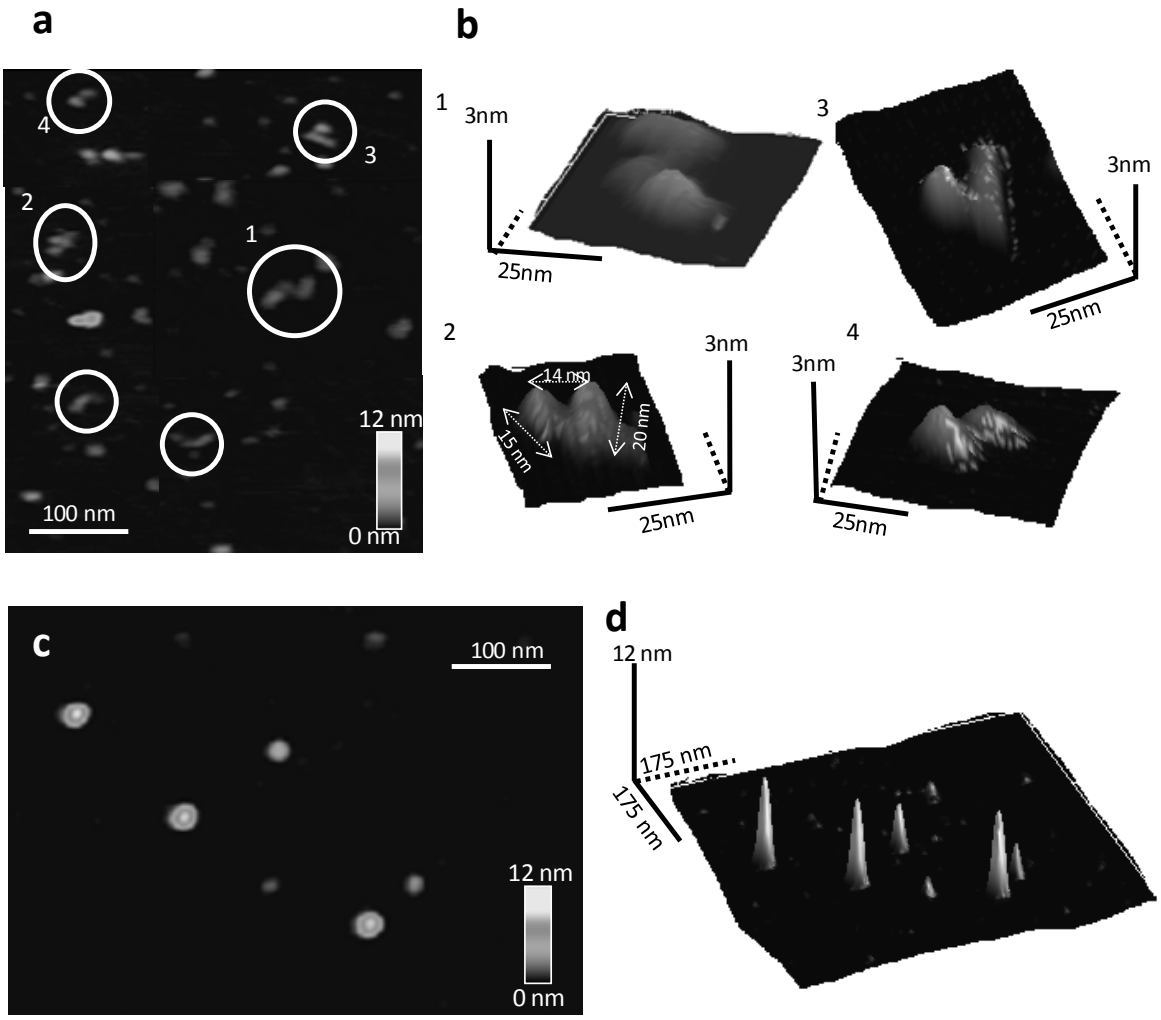

Figure S3 *Single molecule characterization of I27$_2$–AnkR-I27-SecM$^{150–166}$ protein and control sample using AFM.* (a) Shows height AFM images of I27$_2$–AnkR-I27-SecM$^{150–166}$ obtained from *in vivo* expression. (b) Shows magnified images of the structures 1-4 from (a). Example of typical molecule dimensions: length of longer arm = 20nm (18.3 ± 2.2 nm; mean ± s.d. for *n*=25); length of shorter arm = 15 nm (13.7±1.4 nm; *n*=25); end-to-end distance = 14 nm (17.2 ± 2.6 nm; *n*=25) is marked on structure 2. (c) 2D and (d) 3D height AFM images of control sample obtained from *in vitro* expression (no DNA added).