Interfacial self-assembly of a bacterial hydrophobin

Citation for published version:

Digital Object Identifier (DOI):
10.1073/pnas.1419016112

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Proceedings of the National Academy of Sciences

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Interfacial self-assembly of a bacterial hydrophobin

Keith M. Bromley, Ryan J. Morris, Laura Hobley, Giovanni B. Brandani, Matthew McCluskey, Ulrich Zachariae, Davide Marenduzzo, Nicola R. Stanley-Wall, and Cait. E. MacPhee

Supporting information

Materials and Methods

**Protein purification:** The BslA proteins used in this study were purified using an adaptation of the method described in previous work (1). The plasmids encoding overexpression constructs containing bslA<sub>42-181</sub> fused to GST and separated by a tobacco etch virus (TEV) protease recognition site (wild-type bslA: pNW1128 and bslA<sub>L77K</sub>: pNW1189 (1)) were transformed into E. coli BL21 (DE3) pLysS. The transformed cells were grown in LB broth (supplemented with ampicillin (100 µg.mL<sup>-1</sup>)) overnight, then inoculated into autoinduction media (2) (again supplemented with ampicillin (100 µg.mL<sup>-1</sup>)) at a ratio of 1:1000 (vol:vol). The cultures were incubated at 37 °C with shaking at 150 rpm until they reached an optical density at 600 nm of ~0.9, then the temperature was reduced to 18 °C overnight. Centrifugation at 4000 × g for 45 min was used to collect the cells, which were then frozen at -80 °C until needed. The cells collected from 1 litre of culture were resuspended in 25 mL of purification buffer (50 mM HEPES, 250 mM NaCl, pH 7.5) supplemented with Complete EDTA-Free Protease Inhibitors (Roche), before being lysed using an Emulsiflex cell disruptor (Avestin) with pressure at 15000 psi applied three times to each sample. Insoluble cell debris and unlysed cells were removed by centrifugation at 27000 × g for 20 min. 0.75 mL of Glutathione Sepharose 4B (GE Healthcare) beads was added to the cleared lysate from 1 L of cells and placed at 4°C with gentle agitation for 4 h to allow binding of the GST-tagged BslA protein to the beads. The beads and cell lysate was then loaded onto a single-use gravity flow column (Biorad) and the liquid allowed to flow through. The beads were then washed twice by the addition of 25 mL of the purification buffer to remove any unbound proteins. The washed beads were then collected and added to 25 mL of purification buffer supplemented with 1 mM DTT and 0.5 mg of purified TEV protease, and placed at 4 °C overnight with gentle agitation. The solution containing the beads, released BslA, TEV protease and unbound GST was again loaded onto the gravity flow columns and the flow-through collected. To the flow-through 0.75 mL of fresh Glutathione sepharose beads and 0.25 mL of Ni-NTA (nickel-nitrioloacetic acid) agarose beads (Qiagen) were added and the solution incubated at 4°C with gentle agitation overnight to remove the TEV protease and any unbound GST. The mixture was again passed through the gravity flow column and the purified protein collected in the flow-through. The protein was then concentrated using Vivaspin concentrators (Sartorius).

The proteins were then further purified by size-exclusion chromatography using a Superdex 75 10/300 GL column (GE Healthcare); the fractions containing pure BslA were combined and concentrated again using Vivaspin concentrators. When buffer exchange into 25mM phosphate buffer pH 7 was required it was done during the concentration step.
**SEC-MALLS analysis of BslA:** The purified BslA protein was diluted to a concentration of 2 mg.mL\(^{-1}\) in 50 mM HEPES 250 mM NaCl pH 7.5 buffer, and 140 µL was analysed by SEC-MALLS using a Dionex Ultimate 3000 HPLC system containing an inline miniDAWN TREOS multi angle light scattering detector (Wyatt) and an Optilab T-rEX differential refractive index detector (Wyatt). A Superdex 75 10/300 column (GE Healthcare) was washed with 1.4 column volumes of 0.1 M HNO\(_3\), then equilibrated using 50 mM HEPES 250 mM NaCl pH 7.5 buffer and the reference cell for the differential refractive index detector was purged with the same buffer. ASTRA v6.0.0.108 software (Wyatt) was used to calculate the molar masses across the elution peaks.

**SEC analysis of BslA dimer formation:** The purified BslA proteins were diluted to a concentration of 2 mg.mL\(^{-1}\) in 50 mM HEPES 250 mM NaCl pH 7.5 buffer. 0.5 mL of this protein was loaded onto a Superdex 75 10/300 GL column (GE Healthcare) pre-equilibrated in the same buffer. The sample was run at a constant flow rate of 0.5 mL.min\(^{-1}\) and 0.5 mL fractions collected. The central fraction from each peak was collected and kept for reloading onto the column after the specified time. The elution volumes of each peak were compared with those for the standards from the Gel Filtration LMW Calibration Kit (GE Healthcare) to determine their approximate molecular mass.

**SEC for separating monomers and dimers:** To separate BslA into monomer and dimer fractions, BslA was prepared and isolated as described above. Buffer exchange from HEPES buffer (100 mM, pH 7.5, 250 mM NaCl) to phosphate buffer (25 mM, pH 7) was achieved by several cycles of spin concentration (Vivaspin concentrators, Sartorius) and subsequent dilution into phosphate buffer. Size exclusion chromatography was performed using an ÄKTA-10 FPLC instrument equipped with a Superdex 75 10/300 GL column. Samples were run at a constant flow rate of 0.5 mL.min\(^{-1}\) and the collection fraction volume was 0.5 mL.

**SDS-PAGE:** SDS-PAGE analysis of BslA was performed using purified samples of BslA (5 µl and 10 µl samples from a stock solution at 6.75 mg.mL\(^{-1}\)) diluted 1:1 vol:vol in 4X loading buffer (6.2 g SDS, 40 ml 0.5 M Tris pH 6.8, 6.4 ml 0.1 M EDTA, 32ml glycerol, 1 mg Bromophenol blue) either with or without β-mercaptoethanol at 4 % vol:vol. Samples added to loading buffer containing β-mercaptoethanol were boiled at 100 °C for 5 minutes prior to loading on the gel, whilst the samples without β-mercaptoethanol were not subjected to boiling. Samples were run on a standard 14 % polyacrylamide SDS-PAGE at 200 V for 60 min, before staining with Coomassie Blue.

**Dynamic light scattering:** Dynamic light scattering (DLS) was performed using an ALV-5000 spectrometer-goniometer equipped with an ALV/LSE-5004 digital multiple tau correlator and 632.8 nm He-Ne laser. The scattering signal was collected at a detection angle of 90°. All experiments were performed at 20 °C. Data was analysed using ALV-Correlator software; the correlation function was fitted using regularization analysis to yield a size distribution of hydrodynamic radii. Size distribution plots were created using intensity as the y-axis.

**Pendent drop tensiometry:** Monitoring the kinetics of BslA adsorption was achieved using pendant drop tensiometry with drop shape analysis. A Krüss Easydrop tensiometer (Krüss GmbH, Germany) was used in combination with Drop Shape Analysis software. To perform the experiments, a 25 µL droplet containing BslA (25 mM phosphate buffer, pH 7, various concentrations) was ejected from a needle with a 1.84 mm diameter at a rate of 400 µL.min\(^{-1}\) into a covered glass cube. The moment the droplet was fully formed was designated t = 0 sec and drop shape analysis proceeded from that point onwards. A 1500 frame video of the droplet was captured at a frame rate between 2 and 25 frames per second, depending on the timeframe of the experiment. The video was then analysed frame by frame using Drop Shape Analysis software.

Regime I “lag” times were calculated by identifying the onset of the decrease in interfacial tension (IFT), unless an increase in Laplace fit error was observed at an earlier timepoint. To identify the end of Regime I, straight lines were fit through the Regime I and II regions and the intersect of the two lines was denoted as the onset time.
**Preparation refractive index matched emulsions (RIMEs):** BslA-stabilised emulsions were prepared by mixing decane (10 vol%, $n = 1.41$) and BslA solution (0.1 mg.mL$^{-1}$, 25 mM pH 7 phosphate buffer) in a rotor-stator (IKA Ultra Turrax T10) at Level 6 ($\sim$30000 rpm) for 1 minute. Refractive index matching was achieved by replacing the subphase with 59 wt% glycerol ($n = 1.41$), resulting in a translucent emulsion.

**Circular dichroism (CD) spectropolarimetry:** CD was performed using a Jasco J-810 spectropolarimeter. Control samples were analysed at a concentration of 0.1 mg.mL$^{-1}$ (6.7 $\mu$M) in a 0.1 cm quartz cuvette. Refractive index matched emulsions were analysed in a 0.01 cm demountable quartz cuvette. Measurements were performed with a scan rate of 50 nm.sec$^{-1}$, a data pitch of 0.1 nm and a digital integration time of 1 sec. Twenty accumulations were measured and averaged to produce the final curve. The raw data is presented as semi-transparent dotted lines, whereas data smoothed using Savitzky-Golay smoothing is represented by solid lines. Very little change in folding was observed when samples were dispersed in 59wt% glycerol solution.

**Transmission electron microscopy:** BslA-WT and L77K samples were deposited onto carbon-coated copper grids (Cu-grid) (TAAB Laboratories Equipment Ltd) and imaged using a Philips / FEI CM120 BioTwin transmission electron microscope. To assemble BslA at the carbon-water interface, a 5 $\mu$L droplet of BslA (0.025 mg.mL$^{-1}$) was pipetted onto a Cu-grid and left for 5 minutes before being wicked with filter paper from the side. Then, a 5 $\mu$L droplet of 2% uranyl acetate was placed on the Cu-grid and left for 5 minutes before being wicked from the side with filter paper. BslA-WT flakes were prepared by first shearing a solution of BslA-WT (0.1 mg.mL$^{-1}$) in a rotor-stator (IKA Ultra Turrax T10) at Level 6 (30000 rpm). The resultant stabilized air bubbles creamed and the subphase was replaced with phosphate buffer (25 mM, pH 7). The creamed samples were left at 4 °C for several hours until an insoluble sediment was observed. A 5 $\mu$L droplet containing a small amount of sediment was pipetted onto a Cu-grid and allowed to dry. The grid was then stained with 2% uranyl acetate in the same manner as described above.

**Molecular Dynamics (MD) simulations:** In our MD simulations, the topology of BslA was generated by combining the standard MARTINI-2.2P model with an elastic network (3), where harmonic springs are defined between any pair of non-consecutive residues within a certain cut-off distance. The spring constant and cut-off have been parameterised against all-atom simulations with AMBERsb99-ildn (4). The elastic network maintains the correct tertiary structure and large-scale fluctuations of the protein. The MARTINI topologies where created using the martinez script (5) starting from equilibrated all-atom structures of BslA. For chain I, we added the missing residues in the third cap strand from the same section in chain C, and then we performed an all-atom equilibration of the protein in water. The mutations have been created from the correspondent wild type configurations using the program Pymol (6). All simulations have been performed with the software GROMACS-4.6.1 (7) in the NAP$_2$T ensemble with fixed area of the interface, $P_z = 1$ bar and $T = 300$ K. The free energy of adsorption has been computed using the Jarzynski equality (8),(9). The distance along $z$ between the centre of mass of the protein and the centre of the cyclohexane phase was maintained at a target value $zk$ by a harmonic potential with spring constant $K = 10000$ kJ.mol$^{-1}$.nm$^{-2}$. The equilibrium value of the potential is pulled at a speed $v = 0.1$ Å.ns$^{-1}$ in order to displace the protein from its unbiased equilibrium position at the interface into the bulk water phase.
Adsorption dynamics in the presence of a barrier

Here we describe how to model the absorption of WT-BslA onto an interface by diffusion, in the presence of a barrier. The concentration of WT-BslA, \( c \), obeys the diffusion equation,

\[
\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial z^2}
\]

where \( D \) is the protein’s diffusion coefficient and \( z \geq 0 \) is the direction perpendicular to the interface. The boundary conditions are

\[
c(z, t) \to c_0 \quad z \to \infty
\]

\[
D \left[ \frac{\partial c(z, t)}{\partial z} \right]_{z=0} = -kc(0, t)
\]

where \( k \) is a kinetic constant, with the dimensions of a velocity, which models the barrier in a simple way (\( k \to \infty \) corresponds to diffusion-limited adsorption).

To find the analytical form of \( c(z, t) \), we can solve Eqs.(1-) in Laplace space, and then compute the inverse Laplace transform of the solution. If we do this, we obtain

\[
c(z, t) = c_0 \left[ 1 - \text{erfc} \left( \frac{x}{2\sqrt{D t}} \right) \right] + c_0 e^{-\frac{k^2}{D} - \frac{x^2}{2Dt}} \text{erfc} \left( \frac{x}{2\sqrt{D t}} - k\sqrt{\frac{t}{D}} \right),
\]

where the term in square brackets on the right hand side is the diffusion-limited results (\( k \to \infty \)).

We can then compute the surface density of WT-BslA as follows,

\[
\Gamma(t) = \int_0^t D \left[ \frac{\partial c(z, t')}{\partial z} \right]_{z=0} dt'.
\]

Performing the integral, we obtain

\[
\Gamma(t) = 2\sqrt{\frac{D t}{\pi}} - \frac{Dc_0}{k} \left( 1 - e^{-\frac{t}{Dk}} \right) + \frac{Dc_0}{k} e^{-\frac{k^2}{D}} \text{erf} \left( k\sqrt{\frac{t}{D}} \right) - \sqrt{2} \frac{Dc_0}{k} \text{erf} \left( k\sqrt{\frac{2t}{D}} \right).
\]
For $t \gg \frac{D}{k^2}$, this expression simplifies to

$$\Gamma(t) \sim 2\sqrt{\frac{Dt}{\pi}} - \frac{Dc_0}{k} (1 + \sqrt{2}).$$ \hspace{1cm} (7)

Dimensional analysis suggests that $k$ is proportional to $D$, and inversely to the size of the protein under consideration, $\sigma$; furthermore the presence of an energy barrier $\Delta E$ should slow down the dynamics a-la-Kramers, so that we expect

$$k \sim \frac{D}{\sigma} e^{-\frac{\Delta E}{k_B T}}$$ \hspace{1cm} (8)

where $k_B$ is the Boltzmann constant and $T$ is the temperature.

The data in Fig. 2 of the main paper for the WT-BslA can be explained (fall below the theoretical prediction) for a value of $\frac{D}{k} \sim 1$–10 microns, which corresponds to $\Delta E \sim 5$–9 $k_B T$ (with $\sigma \sim 1$–10 nm).
Supplemental Results

**WT-BsIA and L77K form dimers in solution.** Size-exclusion chromatography combined with multi-angle laser light scattering (SEC-MALLS) confirmed that both WT- and L77K-BsIA form dimers in solution. SEC of BsIA solutions clearly revealed that both WT-BsIA and BsIA-L77K solutions kept for 2 days at 4°C contained a mix of monomers and dimers, which was confirmed by MALLS (FIG S1) and SDS-PAGE performed without the addition of reducing agent (β-mercaptoethanol) or heat treatment (FIG S2). The monomer:dimer mass ratio was approximately 4:1. Samples stored at room temperature contained a similar ratio of monomers and dimers.
Figure S1: WT-BslA and L77K form dimers in solution. Size-exclusion chromatography combined with multi-angle laser light scattering (SEC-MALLS) confirmed that both WT- and L77K-BslA form dimers in solution. SEC of BslA solutions clearly revealed that both (a) WT-BslA and (b) BslA-L77K solutions kept for 2 days at 4 °C contained a mix of monomers and dimers, which was confirmed by MALLS (FIG S1). The monomer:dimer mass ratio was approximately 4:1 in both WT-BslA and BslA-L77K samples. Samples stored at room temperature contained a similar ratio of monomers and dimers.
Figure S2: SDS-PAGE of BslA without the addition of BME (left) and with the addition of BME (right). BME reduces the disulphide bond that holds the dimer together. In the absence of a reducing agent, dimer, tetramer and hexamer bands are visible in the gel. Addition of BME removes all multimers from solution. The formation of tetramers and hexamers is feasible as each BslA molecule contains two cysteine residues capable of forming disulphide bonds.
Figure S3: Addition of 1 mM dithiothreitol (DTT) to a 0.3 mg.mL⁻¹ solution of BslA dimers (RH ≈ 3.1 nm), slowly reduced the disulphide bonds of the dimers. After 8 hours incubation with DTT at room temperature, the dimeric BslA had been converted into monomeric BslA (RH ≈ 2.1 nm). The black squares represent individual measurements, while the red line is a Boltzmann fit of the data.
Figure S4: (a) This figure is identical to Figure 2 in the main text except data points for WT-BslA and BslA-L77K dimers at a concentration of 0.03 mg.mL$^{-1}$ have been added. Although it initially appears that the formation of dimers causes an additional adsorption barrier, (b) replotting the data using molar concentration demonstrates that the dimer data points fit on the same lines as the monomer data points. This suggests that the area of adsorption of a dimer is identical to that of a monomer, implying that only one cap from a dimer can bind to the interface.
Figure S5: HydroPro calculations (10, 11) performed on PDB files of monomers and various types of dimer revealed that dimers formed by an end-end arrangement of monomers (either cap-cap, tail-tail or cap-tail) should have a hydrodynamic radius of approximately 3 nm. All other reasonable dimer arrangements were predicted to exhibit a hydrodynamic radius between 2.5 and 2.8 nm. Experimental DLS data revealed the hydrodynamic radius of BslA monomers was 2.2 nm, whereas the hydrodynamic radius of BslA dimers was 3 nm, suggesting that BslA dimers are indeed arranged end to end.
Figure S6: Dynamic interfacial tension response curves of droplets of WT-BsIA (0.04 mg.mL\textsuperscript{-1}) in air. The black lines represent IFT data, while the red lines represent error of Laplace fit (FE). (a) IFT decreased before FE increased, but in (b) FE increased before IFT decreased. For plotting the values reported in Figure 2, the earlier of the two events was always reported.
Supplemental References


