Insights in the Organization of DNA-Surfactant Monolayers Using Cryo-Electron Tomography

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ADDITIONAL LANGMUIR EXPERIMENTS

When the Π-A isotherm of a buffered 3 mg/ml DNA sub-phase was recorded without a surfactant (Figure S1), a small increase in surface pressure was observed upon compression, indicating surface activity of the DNA molecules. However, summation of the isotherms of surfactant on a buffer (a) and of DNA (c) did not yield (d) the recorded Π-A isotherm of the surfactant on a DNA containing sub-phase (b), suggesting that the surface activity of DNA alone does account for the observed large liquid expanded region. It can be speculated that no closed surfactant monolayer is formed at the air water interface, but rather a mixed phase of DNA and surfactant.

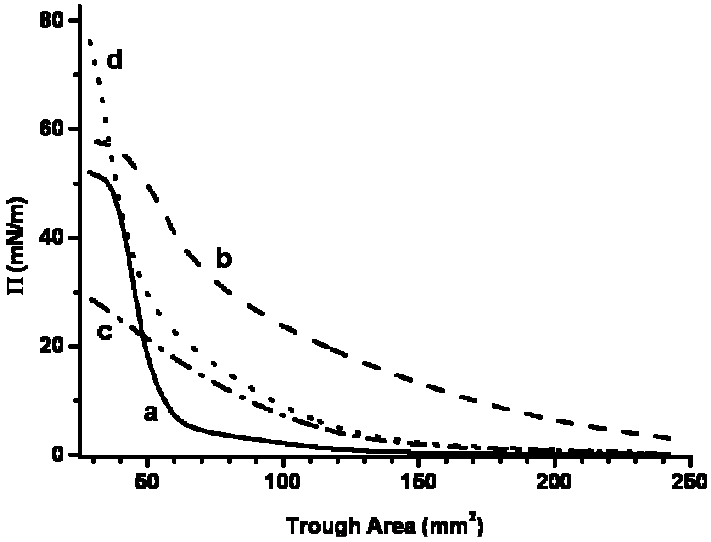

Figure S1. *Π-A isotherms of the compression of surfactant on a PBS buffer* ***(a)(·····)****, surfactant on a 3 mg/ml DNA/PBS subphase* ***(b) (- -)****, a 3 mg/ml DNA subphase* ***(c)(· — ·)*** *and the sum isotherm of* ***a*** *and* ***c*** ***(d) (·····)****, versus the surface of the trough.*

BREWSTER ANGLE MICROSOPY

Since high salt content, as present in PBS buffer, obscures the measurement of the Brewster angle microscope (BAM), water was used as a sub-phase for these measurements. To compare these results with the experiments performed on a buffer sub-phase, Π-A isotherms were also recorded for the bis-ureido based surfactant **1** spread on a water sub-phase (Figure S2A). Upon compression, no increase in surface pressure was detected until a molecular area of ~27 $Å^2$/molecule was achieved. At this point a sudden steep increase in pressure, indicative of a liquid condensed phase, was observed until a collapse was achieve at a surface pressure of 56 mN/m.* Brewster angle microscopy showed the presence of dense domains already at Π = 0 mN/m (Figure S2B). Upon compression to a mean molecular area (MMA) of 26 $Å^2$/molecule, no increase in reflected light intensity for the regions between the domains

was observed (Figure S2A$_I$). Rather, these domains merged upon further compression (Figure S2A$_{II}$). From the point were a continuous dense film was obtained a steep increase in surface pressure was observed. Extrapolation of the slope of the curve in this region to zero pressure represents the MMA of the individual molecules in the corresponding closely packed equilibrium state without external force. The shape of the isotherm and the deduced MMA of 21 Å^2/molecule are similar to the isotherm of surfactant 1 spread on PBS. The sizes and shapes of the domains stay the same up to the point where a continuous layer is formed. Consequently, the head group packing in the self-assembled islands is the same as the MMA deduced from the slope of the surface pressure increase.

Spreading of the bis-urea surfactant on a DNA containing sub-phase (3 mg/ml) and subsequent compression, yielded a completely different isotherm as was expected based on the experiments on the DNA/PBS sub-phase (Figure S2A). At the start of the isotherm, zero pressure was not fully obtained indicating that the lift-off area was above 130 Å^2/molecule. Upon compression a clear liquid expanded phase was observed from ~100 to 35 Å^2/molecule. Below 35 Å^2/molecule the isotherm entered the liquid condensed state. The slope of the isotherm was clearly less steep than in the case of the liquid condensed phase on pure water. Nevertheless, the maximum collapse pressure was significantly higher (66 mN/m). Extrapolation of the liquid condensed phase to zero pressure yielded a MMA of approximately 50 Å^2/molecule. Brewster angle microscopy again showed domain formation already at low surface pressures (~ 4 mN/m), although the domains showed much smoother edges (Figure S2A$_{III}$/C) and appeared to be fewer in number. Upon compression, the domains were pushed together in a similar fashion as on pure water. However, in this case the regions between the domains did show an increased density upon increasing the surface pressure (Figure S2D). This separate phase prevented the domains from fusing at high compression (Figure S2A$_{IV}$). Judging from the extrapolated MMA (48 Å^2/molecule), surfactant molecules cannot form a closed packed arrangement when spread on a DNA sub-phase, which would result in a MMA of ~22 Å^2/molecule.

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[*] No liquid expanded phase, even at elevated temperatures up to 50 °C, could be observed in any of the recorded isotherms.

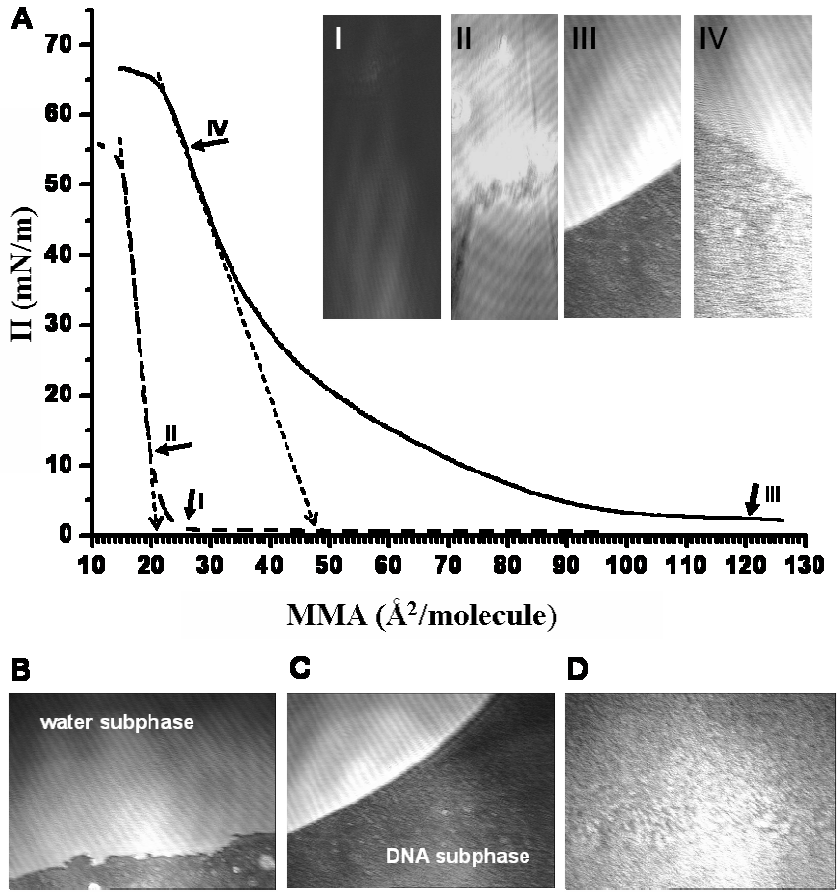

Figure S2. A. *Surface pressure vs. surface area (Π-A) isotherms of bis-urea surfactant **1** spread and compressed on a water **(- -)** and on a DNA containing **(—)** sub-phase [DNA] = 3 mg/ml). The arrows indicate the MMA extrapolated to zero pressure. Inset: Brewster angle microscopy (BAM) images taken at various stages of compression as indicated by the roman numbers in the isotherm.* **B.** *BAM image of a dense domain with rough edges of surfactant **1** spread on a water sub-phase (Π = 4 mN/m).* **C.** *Same as **B** but on a DNA containing sub-phase; note the smooth edge of the domain.* **D.** *BAM image of only the phase between the domains when the bis-urea surfactant is spread on a DNA containing sub-phase and compressed to a surface area of 26 Å^2/molecule (Π = 55 mN/m); note that the density at this surface pressure is still lower than that of the domains at 0 mN/m.*

Also in this case a similar isotherm was obtained when the experiment was performed using an aqueous DNA sub-phase instead of a PBS/DNA sub-phase. Although both experiments using water instead of PBS gave similar results, some differences were observed. Due to screening of the charges caused by pH control and high salt content, the lower repulsion between the charged head groups will result in a lower surface pressure, which explains the observed decrease for the surfactant on a DNA/PBS sub-phase compared to the surfactant on an aqueous DNA sub-phase.

It is also known that highly charged surfactants have a tendency to oppose phase transitions and thus screening of the charge may result in their appearance. The observed early lift-off and subsequent small increase in pressure before the liquid condensed phase for the surfactant on PBS versus the complete absence of a liquid expanded state for the surfactant on water can be the result of this effect.[1]

Injection of an aqueous DNA solution in a PBS sub-phase underneath a preformed monolayer kept at a constant pressure of 35 mN/m again did not result in any change in surface area. In this experiment the DNA solution was completely mixed throughout the sub-phase by 10 minutes flow circulation.[†] These results confirm that in this configuration DNA does not penetrate the monolayer structure.

A hysteresis experiment was performed to establish the stability of the surfactant monolayer. When the compressed monolayer on an aqueous subphase is decompressed in the first expansion cycle to Π = 0 (Figure 3, E_1), the pressure drop is much steeper than the pressure incline of the first compression (Figure 3, C_1), which indicates no expansion of the monolayer, because this would be accompanied by a more gradual drop in surface pressure similar to the pressure incline of the first compression. A second compression cycle (C_2) showed a steep incline that overlaps with the first expansion cycle (E_1), indicating again that, when relaxed after the first expansion, the densely packed islands do not break apart or expand. The MMA that is reached after the second compression is the same (35 mN/m) as in the first compression, which indicates no dissolution of molecules, because this would lead to a smaller

[†] In a separate experiment the mixing was confirmed by using ethidium bromide.

MMA due to loss of surfactant molecules at the air-water interface. The second expansion (E_2) cycle shows again a steeper drop than the first expansion, indicating a similar effect: an immidiate pressure drop when the compression is released. This experiment showed that there is no tendecy of the monolayer to expand upon relaxation.

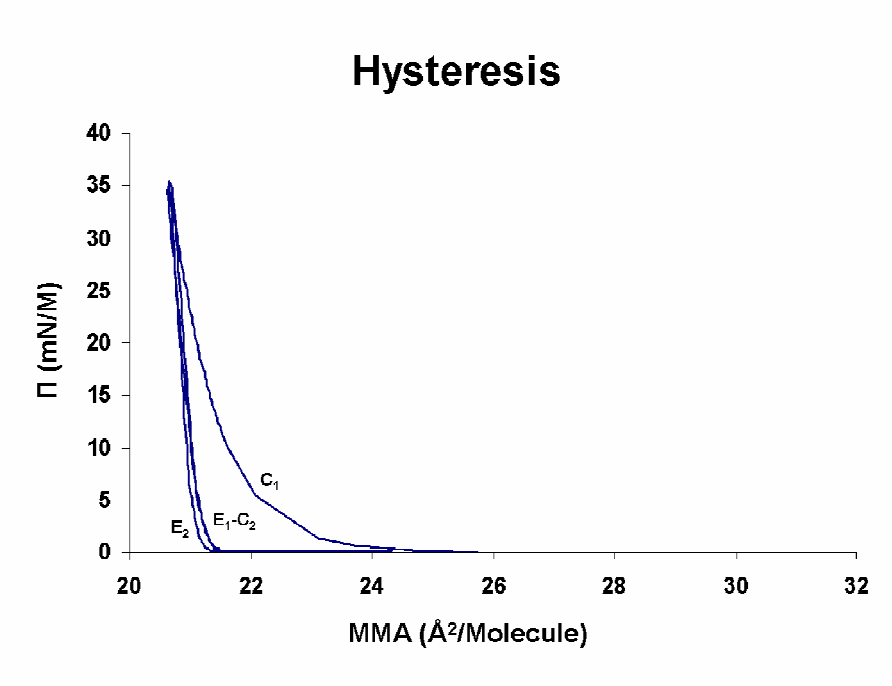

Figure S3. *Π-A isotherm of the compression and expansion of a monolayer of surfactant **1** on an aqueous subphase. C_1 and E_1 denote the first compression-expansion cycle, C_2 and E_2 denote the second cycle.*

CRYO-ELECTRON TOMOGRAPHY

To directly image the DNA-surfactant monolayers using cryo-TEM, a sample preparation method[2] first reported by Leiserowitz *et al.* was adapted (Figure S4). A self-organized monolayer of bis-urea surfactant molecules was formed on the surface of a PBS sub-phase in a glass dish, with a density corresponding to a surface pressure of ~35 mN/m. The dish was situated inside a controlled environment (a humidity and temperature glove box) and placed underneath a fully automated vitrification robot

(Vitrobot™ Mark III). Before spreading of the surfactant, a TEM grid, which supported a holey carbon film, was placed on a stainless steel mesh submerged underneath the air-water interface. During spreading on PBS buffer, the formation of a rigid film on the water surface could be visually confirmed and equilibrated for 10 minutes. Next, an aqueous DNA solution was injected in the PBS buffer sub-phase and mixed for 10 minutes using flow circulation. The injected DNA concentration was chosen such that the final sub-phase concentration was 3 mg/mL, equal to the Langmuir experiments. Subsequently, while maintaining 100% humidity to prevent evaporation, the formed monolayer was lowered onto the carbon grid using a drain mechanism. Inside the glove box, the grid was transferred to the plunging mechanism and raised into the environmentally controlled chamber of the Vitrobot. Next, the grid was blotted and vitrified by plunging into liquid ethane and was analyzed using low dose cryo-TEM at -170 °C.

For the formation of a monolayer on top of a DNA-containing sub-phase, the bis-urea surfactant was spread after injection of the DNA solution and allowed to settle for 10 minutes, after which the sub-phase was drained and the DNA-surfactant monolayer was lowered over the TEM grid.

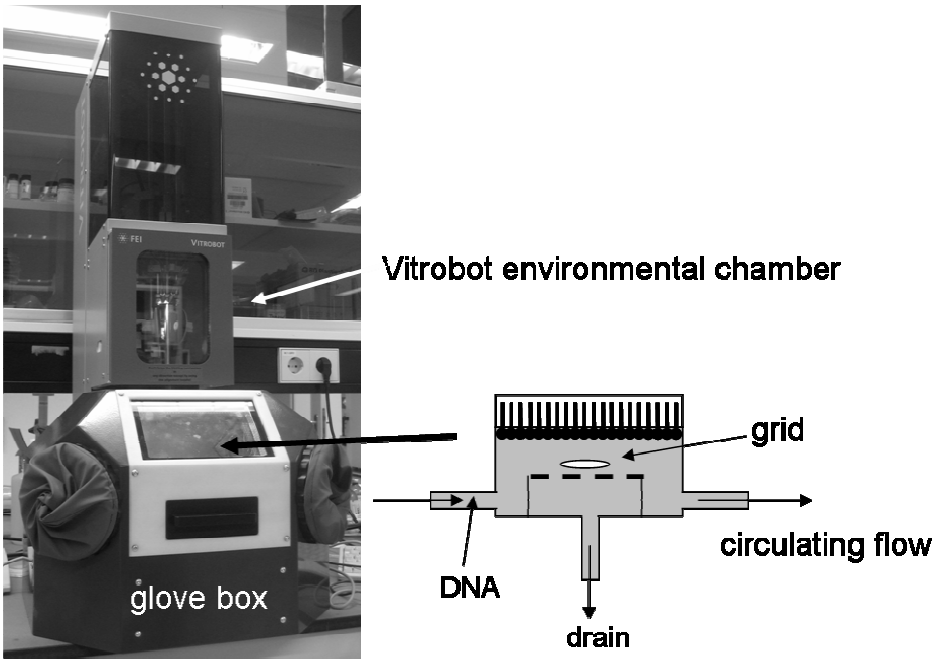

Figure S4. *The Vitrobot fitted with a humidity and temperature controlled glove box, in which a glass dish (depicted in figure S1) is placed. The schematic representation of the dish shows a surfactant monolayer at the air-water interface. A Quantifoil® holey carbon grid is placed underneath the monolayer prior to spreading of the surfactant. A DNA solution can be injected into the circulating flow. DNA is injected before or after surfactant spreading, after which the monolayer can be lowered on top of the grid using a drain mechanism.*

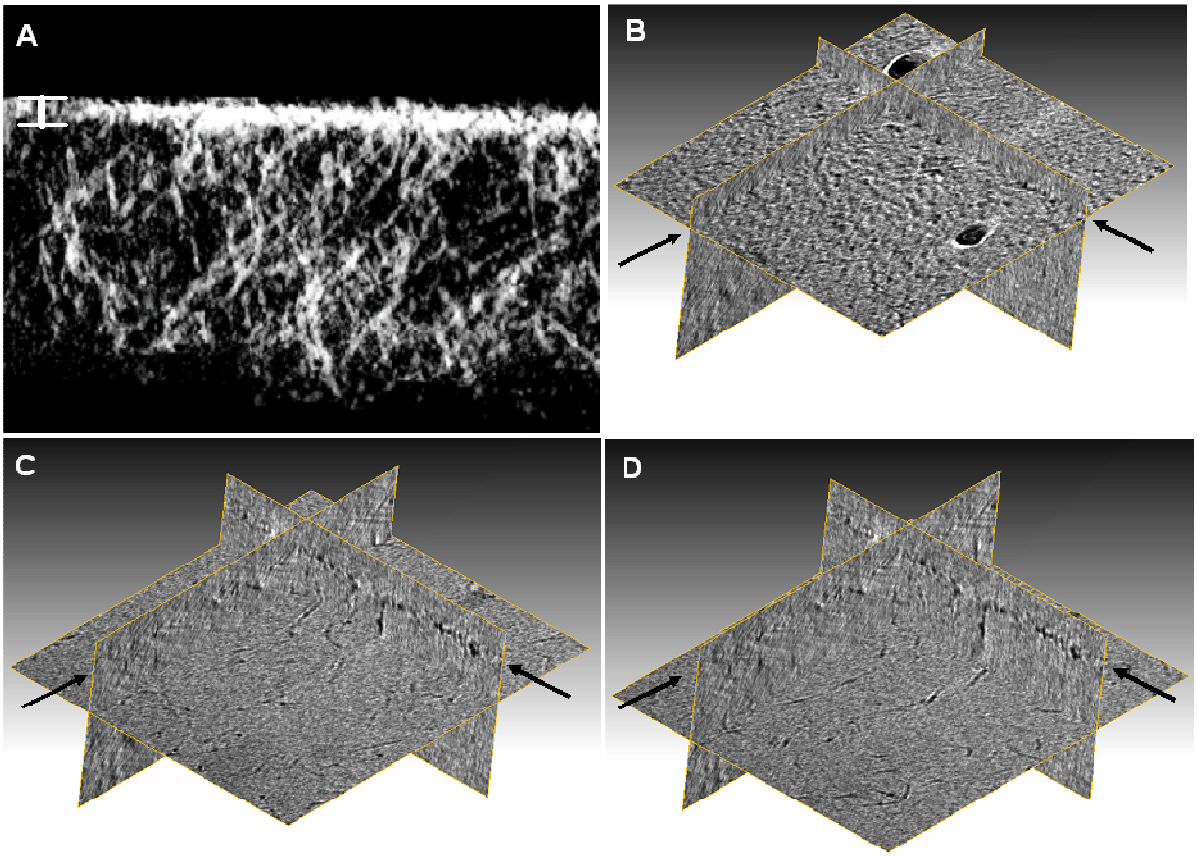

Figure S5. *Reconstruction of a DNA surfactant monolayer, in which DNA was injected underneath a preformed bis-urea surfactant monolayer.* **A.** *Projection along the x- and y-direction of the 3-D reconstructed volume in Amira showing the 2.5 nm DNA strands suspended from the monolayer. Parts of the DNA strands are attached to the cationic monolayer surface resulting in a thin dense layer of DNA at the monolayer surface (indicated by the 10 nm white bar), while the other ends are extending down into the bulk solution.* **B.** *Unprocessed x-, y-, and z-cross-sections combined to form a 3-D volume. The horizontal z-cross-section is positioned at the monolayer surface (indicated by the black*

arrows) and clearly shows the presence of many fibre-like structures in the horizontal plane. **C.** *Same as* ***B*** *with the horizontal cross-section positioned below the monolayer surface. Only a few DNA strands are present when compared to* ***B***. **D.** *Same as* ***C*** *only with the horizontal cross-section positioned lower, showing a similar image as* ***C***.

Vertical cross-sections in the x- and y- direction of the reconstructed volume of the injected DNA-surfactant monolayer revealed a 10 ± 3 nm layer of dense matter present at the top of the reconstructed volume, i.e. at the ice-vacuum interface. Horizontal cross-sections (z-slices) positioned at this layer showed the presence of many fibre-like structures (Figure S5B). When the z-slice is positioned below the dense 10 nm layer, the chain density is significantly decreased and individual DNA strands can be clearly distinguished (Figure S5C/D) comparable to the 3 mg/ml DNA solution in bulk (Communication, Figure 2A). The information from the transmission images of densely packed DNA chains in figure 2A of the communication, originates predominantly from the layer of condensed matter in the top ~10 nm of the vitrified ice layer, i.e. from the air-water interface.

A side view of the total reconstructed 3-D volume clearly shows that the DNA strands are suspended from the surface down into the sub-phase (Figure S5A). Apparently, the individual DNA strands do not bind completely to the monolayer surface and a part of the chain extends down into the sub-phase. Apart from the superposition of all DNA chains present in the total section, this may also explain why complete DNA strands (end-to-end) are not observed in the transmission image presented in figure 2C of the communication.

The absence of beam damage was verified by the aquisition of an image prior to and after the aquisition of a tilt series. As shown in Figure S6, no significant changes can be detected in the cross-correlation of the two images, (apart from some changes in the ice contamination, see arrow) indicating no visual structural changes occurred during aquisition. The total dose of this tilt series was approximately 100 $e^-/Å^2$.

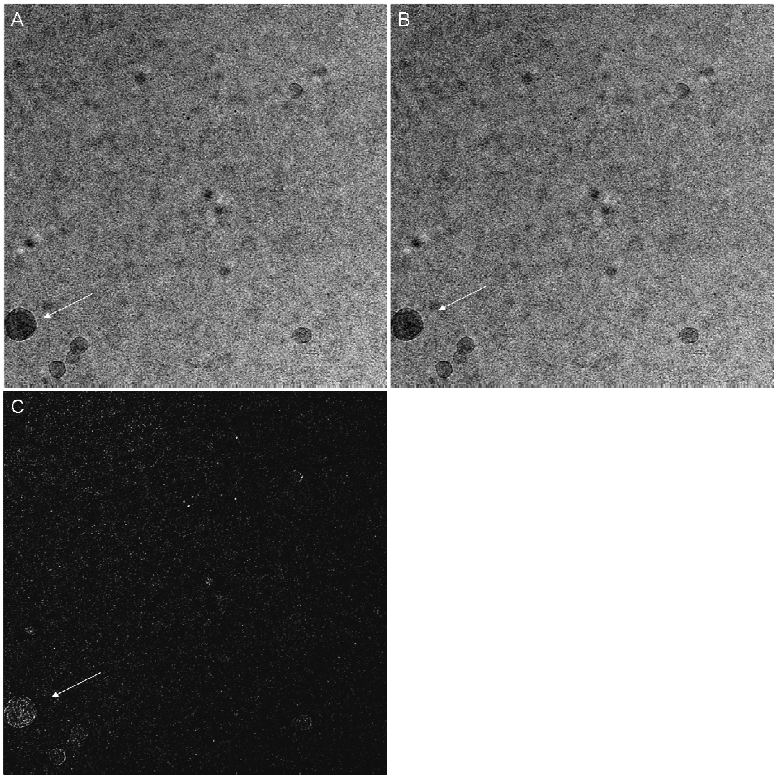

Figure S6. A. *TEM micrograph of the tomography site before aquisition of the tilt series.* **B.** *Same as A, only after aquisition.* **C.** *Cross-correlation between **A** and **B** showing no significant differences between the two images (only an ice contamination, indicated by the white arrow, has changed slightly).*

EXPERIMENTAL

General materials and equipment. Bis-urea surfactant **1** was supplied by The Dutch Spinoza Junior Researchers Institute. DNA (~300 bp/molecule; sodium salt) was kindly provided by Nichiro Corporation (Yokosuka-shi, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the BCA protein assay (Pierce, Rockford, Illinois, USA) and measured to be below 0.20% w/w. Ethidium Bromide was purchased from Bio-Rad. All solvents were purchased from Acros Chemica or Sigma-Aldrich and of p.a. quality. R2/2 Quantifoil Jena grids were purchased from Aurion. PBS buffer was prepared by dissolving 8 g (137mM) NaCl, 0.2 g (2.7 mM) KCl, 1.44 g (10 mM) Na_2HPO_4 and 0.24 g (2 mM) KH_2PO_4 in 800 mL demineralised water, after which the pH was adjusted

to 7.4 using HCl or NaOH solutions and the total volume was brought up to 1L. Ultra pure water was used in all Langmuir experiments (Barnstead EASYpure LF system; R >17.7 MΩ-cm). The Langmuir isotherms were recorded using a KSV 3000 computer controlled system equipped with a temperature controlled removable Minitrough (75.0 x 323 mm) constructed from a solid piece of PTFE. The hydrophilic barriers are made from polyacetal (Delrin). The surface pressure was recorded using a filter paper (10 x 20 mm)(the bis-urea surfactant adsorbs to the platinum of the Wilhelmy plate). The complete KSV 3000 system was situated in a sealed polycarbonate cabinet. Brewster angle microscopy measurements were performed on a KSV Minitrough system equipped with a BAM 300 microscope, which uses a HeNe-laser (10 W, 633 nm) with Glan-Thompson polarizers (10-8 polarization ratio) and a computer controlled high definition CCD camera (768 x 576 pixels). Transmission microscopy measurements were performed on a FEI Titan Krios TEM equipped with a field emission gun (FEG) operating at 300 kV. Images were recorded using a 2k x 2k Gatan CCD camera equipped with a post column Gatan energy filter (GIF). A Gatan cryo-holder operating at ~ -170 °C was used for the cryo-TEM measurements. The sample vitrification procedure was carried out using an automated vitrification robot, viz. a FEI Vitrobot™ Mark III equipped with a humidity and temperature controlled glove box. The Quantifoil grids were treated with a surface plasma treatment using a Cressington 208 carbon coater operating at 5 mA for 40 seconds prior to the sample preparation and vitrification. The tomography reconstruction was performed using the Inspect3D (FEI company) software program version 2.1 and Simultaneous Iterative Reconstruction Technique (SIRT). Additional image analysis was performed using Amira version 3.1.1. Extreme care was taken that all used equipment related to monolayer experiments was thoroughly cleaned.

Langmuir experiments. A 1 mg/mL chloroform solution of bis-urea surfactant **1** was prepared by accurately weighing 10 mg of **1** into a closed vial and adding 8 mL of chloroform, followed by heating to 60 °C and sonication for 5 min. to ensure complete dissolution. After cooling to room temperature, the solution was transferred quantitatively to a 10 mL volumetric flask and brought up to volume. In

general, 25 μL was spread unless indicated otherwise. The surfactant was spread on either water, PBS buffer or a 3 mg/ml DNA solution dissolved in either water or PBS, after which the system was compressed with a 5 mm/min compression rate.

The isotherms of a DNA solution injected underneath a compressed monolayer were recorded by spreading 35 μL on top of a water or PBS sub-phase. Subsequently, the system was compressed to and maintained at a surface pressure of 35 mN/m. A peristaltic pump was used to create a circular flow of DNA underneath the monolayer surface (rate: 8 mL/min), with each end of the tubing, which contained a DNA solution (calculated to a total DNA subphase concentration of 3 mg/mL), positioned underneath one of the barriers. A stained DNA solution with Ethidium Bromide showed complete and homogenous mixing of the pink colour throughout the sub-phase in the case of a PBS sub-phase. When the pink solution reached the monolayer surface no significant change in MMA could be observed at constant surface pressure.

The hysteresis experiment was performed by spreading 35 μL on top of a water subphase, followed by two subsequent compression and expansion cycles with a foreward and backward barrier speed of 5 mm/min. The target surface pressure upon compression was 35 mN/m, similar to the surface pressure used in the cryo-TEM experiments.

BAM measurements. Brewster angle microscopy measurements were performed on monolayers of surfactant **1** spread on a water and a 3 mg/mL DNA sub-phase in water. The experiments were performed using a KSV Minitrough system equipped with a BAM following the same procedure as described above. During compression, a BAM image was recorded every 10 seconds, after which the image was coupled by the BAM software to the corresponding MMA of the recorded isotherm.

Transmission electron microscopy

Conventional cryo-electron microscopy. The cryo-TEM images of the 3 mg/mL DNA solution was obtained by applying small aliquots (3 μl) of the viscous solution to a Quantifoil grid (R2/2 Quantifoil Jena) within the environmental chamber (relative humidity 100%) of the Vitrobot™ at 22 °C. Excess

liquid was blotted away with filter paper using an automatic blotting device within the environmental chamber of the Vitrobot™.[3] The grid was subsequently shot through a shutter into melting ethane placed just outside the environmental chamber. The vitrified specimens were stored under liquid nitrogen and observed at -170 °C. Images were recorded using low-dose conditions.

Monolayer cryo-electron microscopy. The glass dish was placed in a temperature a humidity controlled glove box underneath the environmental chamber of the Vitrobot. The dish and connected tubing of the circular peristaltic pump (with a total volume of 54 ml) were filled with 40 mL PBS buffer and air was removed from the system. A bend metal mesh was placed below the air-water interface and hydrophilic Quantifoil grids were placed on top. Care was taken that the tubing connecters and the grid were situated well below the water line. A 1 mg/mL chloroform solution of surfactant **1** was spread, calculated to a total surface pressure of 35 mN/m and allowed to equilibrate for 10 min. During mixing (8 ml/min), 13 ml of an 11.5 mg/ml DNA solution in water was slowly injected into the tubing. After injection, flow circulation was continued for 10 min. to ensure complete mixing of the DNA with the sub-phase solution, after which the monolayer was slowly lowered onto the Quantifoil grid using a draining tube at the bottom of the dish. While at 100% humidity, the grid was attached to the plunger mechanism and raised into the environmental chamber of the Vitrobot. Subsequently, the glove box was removed and replaced by a tray containing liquid ethane at its melting temperature (-170 °C). After blotting, the grid was shot through a shutter into the melting ethane. The vitrified specimens were stored under liquid nitrogen and observed using low-dose conditions.

For the monolayer spread on a DNA sub-phase the same procedure was used, with the exception that the DNA was injected and mixed during 10 min. before the bis-urea surfactant was spread. After spreading on the DNA sub-phase, the monolayer was also allowed to equilibrate for 10 min., after which the draining and vitrification procedure was started.

A noice reduction algorithm (Nonlinear Anisotropic Diffusion)[4] was applied to the transmission images in Figure 3.

Cryo-electron tomography. The samples as prepared in the above section were also used for low-dose cryo-TEM tomography; 300 µL of anionic gold tracer suspension (Ø 15 nm, Aurion) was added to the injected DNA solution for feature tracking during the reconstruction procedure. The region of interest was selected and placed in optimal position for a maximal tilt angle to both sides. The tilt series was recorded over 86 images from +70 to – 70 degrees with a 2° interval between +54 and -54 degrees and a 1° interval for the high tilt angles. Images were taken under low-dose conditions (total dose tilt series ~100 $e^-/Å^2$) with a 3.9 Å pixel size on the CCD camera using the FEI Xplore3D software.

REFERENCES

(1) (a) Binks, B. P. *Adv. Colloid Interface Sci.* **1991**, *34*, 343, (b) Harkins, W. D. *The physical chemistry of surface films*; Reinhold: New York, 1952, (c) Roberts, G. *Langmuir-Blodgett films*; Plenum Press: New York, 1990.

(2) Majewski, J.; Margulis, L.; Jacquemain, D.; Leveiller, F.; Böhm, C.; Arab, T.; Talmon, Y.; Lahav, M.; Leiserowitz, L. *Science* **1993**, *261*, 899-902.

(3) Frederik, P. M.; Hubert, D. H. W. *Methods Enzymol.* **2005**, *391*, 431-448.

(4) Frangakis, E.S.; Hegerl, R. *J. Struc. Biol.* **2001**, *135*, 239-250.