Supporting Information

S2 Subunit of SARS-CoV-2 Spike Protein Induces Domain Fusion in Natural Pulmonary Surfactant Monolayers

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Experimental Section

Materials. Infasurf was a gift from ONY Biotech (Amherst, NY). It was prepared from the lung lavage of newborn calves by centrifugation and organic extraction. Infasurf contains most phospholipids and neutral lipids, mainly cholesterol, of the natural PS, as well as two hydrophobic surfactant proteins, SP-B and SP-C. The hydrophilic surfactant proteins, SP-A and SP-D, however, were removed during the extraction process. Infasurf was stored in sterilized vials with an initial phospholipid concentration of 35 mg/mL. It was diluted to 1 mg/mL using phosphate-buffered saline (PBS) on the day of experiments. For the study of spread monolayers, Infasurf was extracted with chloroform-methanol, dried under a nitrogen stream, and re-suspended in chloroform to a final concentration of 1 mg/mL.

The recombinant SARS-CoV-2 spike S2 subunit protein was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. It was produced by the Baculovirus-Insect Cells expression system and expressed with sequence Ser686-Pro1213 of the SARS-CoV-2 spike S2 ECD fused with a His-tag at the C-terminus. The S2 subunit protein was received in the form of lyophilized powder, and was dispersed in Milli-Q water to form a 1 mg/mL stock solution.

Constrained Drop Surfactometry (CDS). CDS is a new generation of droplet-based surface tensiometry technique developed in our laboratory. It uses the air-water surface of a sessile drop (≈3 mm in diameter, ≈0.3 cm² in surface area, and ≈20 μL in volume) to accommodate the spread or adsorbed surfactant film. As shown in Figure 1C, a key design of the CDS is a carefully machined pedestal that uses its knife-sharp edge to prevent film leakage even at very low surface tensions. System miniaturization of the CDS facilitates rigorous control of the experimental conditions with an environmental control chamber. The spread/adsorbed film at the droplet surface can be compressed and expanded by precisely oscillating the surface area of the droplet using a
motorized syringe. The surface tension and surface area of the film were determined with closed-loop axisymmetric drop shape analysis (CL-ADSA) by analyzing the shape of the film-covered droplet. The surface pressure ($\pi$) can be calculated from surface tension ($\gamma$) using $\pi = \gamma_0 - \gamma$, where $\gamma_0$ is the surface tension of the clean surfactant-free air-water surface.

To study the biophysical impact of the S2 subunit protein on Infasurf, dynamic compression-expansion cycling of the Infasurf film was conducted with CDS at physiologically relevant temperature and humidity, i.e., 37 °C and close to 100% relative humidity. The detailed experimental protocol can be found in our previous publication. Briefly, Infasurf was diluted to a concentration of 1.2 mM and mixed with the S2 subunit to a final concentration of 0.0017 mM or 0.0085 mM, i.e., 0.15 mol% and 0.75 mol% of Infasurf. Mixtures of Infasurf and the S2 subunit were incubated at 37°C for 10 minutes prior to dynamic cycling. A 7 μL droplet of the mixture was dispensed onto the CDS pedestal, and allowed 5 min for adsorption, as indicated by reaching the equilibrium surface tension of approximately 22 mN/m. The Infasurf film was then compressed and expanded at a rate of 3 s/cycle with a controlled 20% compression ratio (CR), to simulate normal tidal breathing. To obtain the absolute minimum surface tension of the Infasurf film, overcompression at 30% and 40% CRs was also studied. At least ten continuous compression-expansion cycles were studied for each droplet. Dynamic surface activity was quantified with the minimum surface tension ($\gamma_{\text{min}}$) at the end of compression, and the average isothermal film compressibility $\kappa_{\text{comp}} = \frac{1}{A} \left( \frac{\partial A}{\partial y} \right)_T$. The maximum surface tension ($\gamma_{\text{max}}$) and the film compressibility during expansion ($\kappa_{\text{exp}}$) of the Infasurf film were also recorded. All results were shown as mean ± standard deviation ($n > 3$). One-way ANOVA with the Tukey means comparison test was used to determine group differences (OriginPro, Northampton, MA). $p < 0.05$ was considered to be statistically significant.
To study the effect of the S2 subunit protein on the spread Infasurf monolayer, ~0.05 µg extracted Infasurf samples were spread onto the air-water surface of a ~10 µL water droplet. The volume of the droplet was subsequently increased to ~19 µL, and the droplet was left undisturbed for 5 minutes to allow evaporation of chloroform and to reach equilibrium. 1 µL stock solution of the S2 subunit protein was injected into the droplet to reach a final protein concentration of 0.05 mg/mL. The spread Infasurf monolayer was then compressed at a quasi-static compression rate of 0.1 cm²/min. The environmental temperatures were controlled at 20 °C and 37 °C, respectively.

**Atomic Force Microscopy (AFM).** The lateral structure and topography of the Infasurf monolayer were studied at the characteristic surface pressure of 30 mN/m. The Infasurf monolayer was first Langmuir-Blodgett (LB) transferred from the droplet by lifting a small piece of freshly peeled mica sheet at a speed of 1 mm/min. Topographical images of the immobilized Infasurf monolayer were obtained with an Innova AFM (Bruker, Santa Barbara, CA). Samples were scanned in air with the contact mode using a silicon nitride cantilever with a spring constant of 0.12 N/m and a tip radius of 2 nm. Each sample was scanned at multiple locations to ensure representativeness and reproducibility. AFM images were analyzed using Nanoscope Analysis (version 1.5).
Run a Run b Run c

Infasurf (CR = 20%)

Infasurf + 0.15 mol% S2 protein (CR = 20%)

Infasurf + 0.75 mol% S2 protein (CR = 20%)

Infasurf (CR = 30%)

Infasurf + 0.15 mol% S2 protein (CR = 30%)

Infasurf + 0.75 mol% S2 protein (CR = 30%)

Infasurf (CR = 40%)
Figure S1. Reproducibility of the dynamic cycling experiments for Infasurf with and without the S2 subunit upon three different film compressions of 20%, 30%, and 40%.
Figure S2. Effects of 0.1 mg/mL subphase-injected S2 subunit on the compression isotherms of Infasurf at 20 °C, with a monolayer compression rate of 0.3 cm²/min.
Figure S3. Adsorption kinetics of 0.1 mg/mL S2 subunit determined at 20 °C. The equilibrium surface tension of the S2 subunit was found to be approximately 50 mN/m.
**Figure S4.** Lateral structure of the Infasurf monolayer at the surface pressure of 30 mN/m under 20 °C. The AFM images were acquired at three different scanning sizes, *i.e.*, 50×50, 20×20, and 10×10 μm. The z range for all images is 5 nm.
**Figure S5.** Lateral structure of the Infasurf monolayer with 0.05 mg/mL subphase-injected S2 subunit at the surface pressure of 30 mN/m under 20 °C. The AFM images were acquired at three different scanning sizes, *i.e.*, 50×50, 20×20, and 10×10 μm. The z range for all images is 5 nm.
Table:

| Scanning Size | Image 1 | Image 2 | Image 3 |
|---------------|---------|---------|---------|
| 50×50 μm      | ![Image 1](image1.png) | ![Image 2](image2.png) | ![Image 3](image3.png) |
| 20×20 μm      | ![Image 4](image4.png) | ![Image 5](image5.png) | ![Image 6](image6.png) |
| 10×10 μm      | ![Image 7](image7.png) | ![Image 8](image8.png) | ![Image 9](image9.png) |

Figure S6. Lateral structure of the Infasurf monolayer at the surface pressure of 30 mN/m under 37 °C. The AFM images were acquired at three different scanning sizes, i.e., 50×50, 20×20, and 10×10 μm. The z range for all images is 5 nm.
Figure S7. Lateral structure of the Infasurf monolayer with 0.05 mg/mL subphase-injected S2 subunit at the surface pressure of 30 mN/m under 37 °C. The AFM images were acquired at three different scanning sizes, i.e., 50×50, 20×20, and 10×10 μm. The z range for all images is 5 nm.
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