The Influence of Lipids on the Interfacial Disposition of Respiratory Syncytical Virus Matrix Protein

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Supplementary Experimental Section: Full Methods

General. All chemicals were used as received unless stated otherwise. All solvents used were reagent grade from Fisher Scientific, Loughborough, UK and used without further purification. Water was purified using a Milli-Q 5 system (Millipore, Billerica, MA, USA) to give a resistivity of 18.2 MΩ. Phosphate buffered saline (PBS), pH 7.4, was obtained from Invitrogen, Paisley, UK at 10 × concentration (10.6 mM KH_2PO_4, 30 mM $Na_2HPO_4.7H_2O$, 1.55 M NaCl), diluted with Milli-Q water and adjusted to the correct pH using HCl or NaOH. All lipids were obtained as lyophilized solids. DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) was purchased from Bachem AG, Bubendorf, Switzerland, while DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) were Sigma brand (Sigma-Aldrich Ltd., Dorset, UK). Sphingomyelin from chicken egg yolk was Fluka brand (Sigma-Aldrich). Cholesterol was purchased from Lancaster (Alfa Aesar, Heysham, Lancashire, UK). Prior to use lipids were dissolved in chloroform or 2:1 chloroform: methanol and stored at -20 °C. RSV M protein bearing a single point mutation (M254 to R) was used for binding experiments, prepared as described previously.[1] Matrix protein samples were stored at 4 °C and used in PBS at pH 7.4. Prior to each experiment, the protein sample was centrifuged at 100,000 × g for 30 min at 4 °C to remove nucleation centers and the protein concentration re-determined.

Tensiometry. A dual-barrier Langmuir trough (102M, Nima Technology Ltd, Coventry, UK) was used for all tensiometry. Lipid films were spread in chloroform, equilibrated for 15-20 min and subjected to 3 cycles of half-compression/expansion. The final isotherm was recorded at a barrier speed of 9 cm^2 min^{-1}. For isotherms with RSV M, the protein was added to the surface prior to the spreading of the lipid.
The surface pressure, Π was calculated from the surface tension, γ, according to eq S1:

$$\Pi = \gamma_0 - \gamma \qquad \text{(S1)}$$

where γ_0 is the observed surface tension at the air-water interface in the absence of protein. For protein adsorption isotherms, the surface tension data were modelled using a four-parameter logistic equation, eq S2: [2]

$$\gamma_c = \left(\frac{\gamma_w - \gamma_l}{1 + (\ln c_P^{\Pi/2} / \ln c_P)^B} \right) + \gamma_l \qquad \text{(S2)}$$

where γ_c is the calculated interfacial tension, γ_w and γ_l are the horizontal asymptotes, $c_P^{\pi/2}$ is the protein concentration for half-maximal change in interfacial tension and B is a factor that describes the gradient of the linear part of the curve. The limiting surface pressure Π^{max} (*i.e.* $\gamma_w - \gamma_l$) was determined from the fitted parameters. Although non-linear least squares fitting was conducted using eq S2, the data are more conveniently presented (as for the solid line in Figure 1 *a*, in the main paper) in terms of surface pressure, calculated according to eq S3:

$$\Pi_c = \gamma_w - \gamma_c \qquad \text{(S3)}$$

where Π_c is the calculated surface pressure.

The apparent Gibbs surface excess (Γ_a) was determined from the gradient of the linear part of the sigmoidal curve (eq S4)[3,4] and the concentration required to produce the maximum change in interfacial tension (c_P^{max}) obtained by extrapolation of the linear part of the curve to $\gamma = \gamma_l$.

$$\Gamma_a = \frac{1}{RT} \bullet \frac{d\Pi}{d \ln c_P} \qquad \text{(S4)}$$

Adsorption of M to lipid monolayers. Adsorption equilibrium constants were determined by taking sections through the Π-A isotherms at constant area, in a region of the data in which transitions or collapse were absent at all protein concentrations. In practice, this was at large areas per lipid molecule (Figure S3). The pressure *vs* concentration data were then modelled using a modified Langmuir isotherm.[2,5]

The surface coverages of protein, θ_P and lipid, θ_L are given by:

$$\theta_p = \Gamma_P / \Gamma_{P,max} \qquad \text{(S5)}$$

$$\theta_L = \Gamma_L / \Gamma_{L,max} \qquad \text{(S6)}$$

where Γ_P and Γ_L are the observed surface excesses of protein and lipid respectively, and $\Gamma_{P,max}$ and $\Gamma_{L,max}$ are the corresponding maximal surface excesses at surface saturation.

The constants ω_L and ω_P may be defined, where:

$$\omega_L = 1/\Gamma_{L,max} \qquad \text{(S7)}$$

$$\omega_P = 1/\Gamma_{P,max} \qquad \text{(S8)}$$

Isotherms were calculated using the Pethica equation[5] (eq S9):

$$Kc_P = \frac{\theta_P}{1 - \theta_L - \theta_P} \qquad \text{(S9)}$$

where K is the adsorption equilibrium constant. θ_L was calculated directly from the area per lipid molecule and ω_L. θ_P was calculated by rearranging eq S9:

$$\theta_P = \frac{Kc_P(1 - \theta_L)}{1 + Kc_P} \qquad \text{(S10)}$$

The pressure change on protein adsorption, $\Delta\Pi$, depends on the fractional occupancy of the surface by the protein, θ_P according to eq S11:

$$\Delta\Pi = \theta_P \Delta\Pi^{max} \qquad \text{(S11)}$$

where $\Delta\Pi^{max}$ is the limiting surface pressure change.

eqs S5-S11 give reasonable fits to the data in most cases (Figure S3). The model assumes that the protein and lipids behave as ideal adsorbates and that the surface activities of both proteins and lipids are unaffected by adsoprtion of the protein to the interface. For monolayers with a single lipid component, the adsorption constants are a reasonable indication of the affinity of the protein for the interface. For monolayers composed of lipid mixtures, eqs S5-S11 will only be valid if there are no significant interactions between the different lipid components and the interactions of the protein with each lipid component are the same. Given the surface topography and Brewster angle microscopy (BAM) data for the protein with PC lipids in the absence of SM, which demonstrate that uniform mixtures are obtained, it is reasonable to use eqs S5-S11 to fit the data. In the absence of more information on the mixing behaviour of these protein-lipid mixtures, the adsorption equilibrium constant will only be semi-quantitative, but nevertheless still be a useful parameter for quantifying the interfacial activity of RSV M.

Preparation and imaging of protein-lipid films. Silicon (111) wafers (Cemat Silicon S. A., Warsaw, Poland, 1 cm^2) were cleaned by immersion in a 3:1 (v/v) mixture of 30% H_2O_2 and concentrated H_2SO_4

for 45 min at 80 °C, followed by treatment with a 1:1 (v/v) mixture of 30% H_2O_2 and concentrated HCl at room temperature for 15 min. The clean wafers were rinsed with Milli-Q water, covered and oven dried being treated with a 1 mM solution of octadecytrichlorosilane (OTS, Acros organics) in dry toluene for 36 h. The wafers were sonicated successively in toluene and chloroform (each 2 × 15 min), before a final rinse with Milli-Q water. Protein-lipid films were spread on a PBS subphase in teflon dipping trough (Nima Technology Ltd., Coventry, UK, model number 612D). The OTS-coated wafer was dipped downwards through each film at constant pressure and released into a clean sample vial submerged in the subphase below the dipping mechanism. The top few millimetres of liquid in the vial were removed under suction and glutaraldehyde (Fluka *BioChemika,* 2.6 M in water) added to a final concentration of 250 mM. After 2 min sodium borohydride (1 M solution in 0.2 M sodium hydroxide, 0.14 molar equivalents with respect to glutaraldehyde) was added. AFM imaging was carried out in tapping mode on a MultiMode AFM equipped with a Nanoscope IV controller (Veeco Instruments Inc., California, USA) using etched silicon probes with a spring constant of 40 N/m (Budget Sensors Tap300, Windsor Scientific, Slough), and a drive frequency of 300 kHz. Images were recorded at typical scan rates of 0.5-1.0 Hz, with integral and proportional gains adjusted for optimum imaging (integral ~0.2-0.4, proportional ~0.3-0.5). The amplitude setpoint was typically 1.5-1.6. Each image was recorded with 256 scan lines. Images were analysed using Nanoscope version 6.1.3 software (Veeco Instruments Inc., California, USA). Root mean square roughness (R_q) and average roughness (R_a) values were determined on scans of size 5 μm × 5 μm in all cases.

Brewster angle microscopy. A dual-barrier Langmuir trough (model 102M, Nima Technology Ltd., Coventry, UK) was modified by replacing the window in the centre of its base with a cylindrical glass well approximately 3 cm deep in order to prevent the reflection of excess light passing through the film surface. Light from a polarized 633 nm helium-neon laser (CVI Melles-Griot, Leicester, UK) was passed through a Glan-Taylor polarizer and adjusted so that the beam was incident on the film surface at an angle of approximately 53° to the normal; the angle was then adjusted until minimal reflection was observed from a clean liquid surface. Monolayers were spread as described for the recording of isotherms and observed by monitoring the resulting reflected light entering a CCD video camera (Optem Zoom 70, Qioptik Imaging Solutions, Newport, NY, USA) connected to a PC for the capture of still images using a program purpose-written in LabView (National Instruments Corporation, Austin, TX, USA).

Formation of protein assemblies. A thin film of lipids was prepared on the side of a round bottomed flask by evaporation of a chloroform solution of the lipids. This was dried in a desiccator overnight, and then re-hydrated with a suitable volume of protein in PBS at pH 7.4. The resulting suspension was incubated at 37 °C for 5-7 days, followed by 2 days at 4 °C. Samples were examined by transmission electron microscopy (TEM) using a Hitachi TEM-H7600 transmission electron microscope (Hitachi, Maidenhead, UK), with an accelerating voltage of 80-100 kV. Samples were loaded on 400 mesh carbon film coated copper grids (Agar Scientific, Stansted, Essex) and stained using 1% uranyl acetate. For samples where enrichement by sucrose gradient flotation was conducted, protein/lipid complexes were dialysed against low salt buffer (LSB, 50 mM Tris pH 7.5, 25 mM KCl, 5 mM $MgCl_2$). To 0.5 ml of the mixture, 3.5 ml of 70% (w/v) sucrose in LSB was added with thorough mixing. This was placed in the bottom of a 13 ml centrifuge tube, overlaid sequentially with 55% (w/v) sucrose and 10% (w/v) sucrose in LSB. The gradient was centrifuged for 16 h at 4 °C in a SW41 rotor (Beckman) at 250,000 g, fractionated into 1 ml aliquots. The aliquots were then tested by western blot analysis for the presence of protein using a monoclonal reagent raised agasinst RSV M protein.[6] Low density fractions of the gradients containing protein were diluted in LSB buffer to a final volume of 4 ml and overlaid onto a 1 ml glycerol cushion and centrifuged in an SW60 rotor at 250,000 g for 2 h at 4 °C. The protein was removed from the top of the layer before dialysis against PBS and analysis by EM (details as above).

Protein concentration determination

A portion of volume V (typically ~5 μl) of the protein solution being assayed was added to an aqueous

solution of guanidine hydrochloride (6 M) to make a solution of total volume V_T. The absorbance, A, of this protein solution was determined at 280 nm against a blank of aqueous guanidine hydrochloride (6 M). This was repeated over a range of values for V (typically 4 measurements) at constant V_T and the concentration of the protein stock (c_P) determined by linear fit to eq S12 with A as the dependent variable and V as the independent variable:

$$A = \varepsilon c_P l \frac{V}{V_T} \qquad \text{(S12)}$$

where ε is the extinction coefficient of the protein at 280 nm (calculated[7] as 24,220 $M^{-1}cm^{-1}$) and l is the path length of the cuvette in cm.

Generation of wild-type M protein. The codon encoding arginine at position 254 of the M protein ORF was mutagenised to methionine using the Quik Change mutagenesis kit following manufacturer's instructions (Stratagene (Cheadle, UK), primer sequences available on request). The sequence of the complete ORF was determined and there were no other changes introduced. The methionine changes the M protein to the residue normally found at this position. The wild type protein, M^{254M} was expressed and purified as previously[1] described for M^{254R}. Testing of M^{254M} in assays similar to those described earlier[1] demonstrated that it acts identically to M^{254R}.

Crystallisation and X-ray data collection. Crystals of M^{254M} were grown by sitting drop vapour diffusion according to the previously published method. The flattened needle shaped crystals were harvested in a rayon fibre loop, bathed in a cryo-protectant, produced by supplementing the mother liquor with 30% (v/v) glycerol, and flash frozen in liquid N_2. Data were collected on the in-house copper rotating anode source (Rigaku Micromax 007HF (Rigaku, Sevenoaks, UK)) at a wavelength of 1.54179 Å over an oscillation range of 145° with a $\Delta\phi$ of 0.5° using a Mar345 image plate detector. All data were indexed and integrated in MOSFLM;[8] all other computing was carried out in the CCP4 suite unless otherwise stated.[9] Crystals of M^{254M} were found to crystallise in the monoclinic space group C2 with unit cell parameters of a = 52.50 Å, b = 81.10 Å, c = 65.52 Å and β = 96.12° with one molecule in the asymmetric unit.[10] The structure was solved by molecular replacement using the previously published M^{254R} structure as a starting model. 5% of the native data were set aside for cross validation analysis and the behaviour of R_{free} was used to monitor and guide the refinement protocols. ARP/wARP[11] in conjunction with REFMAC was used to automatically build the sequence into the electron density.[12] Refinement was undertaken in REFMAC with manual correction to the model using COOT.[13] Coordinates and observed structure factors have been deposited with the Protein Data Bank. X-ray data and refinement quality statistics are shown in Table S1.

Table S1. Data collection and refinement statistics for native human RSV M protein (M^{254M}).

Data collection	
Space group	C2
Cell dimensions	
a, *b*, *c* (Å)	52.50, 81.10, 65.52
α, β, γ (°)	90.0, 96.12, 90.0
Resolution (Å)	26.05-1.85 (1.95-1.85)[a]
R_{merge}	0.083 (0.52)
$I / \sigma I$	15.4 (2.6)
Completeness (%)	98.0 (98.0)
Redundancy	3.7 (3.4)
Refinement	
Resolution (Å)	26.05-1.85 (1.95-1.85)
No. reflections	22370
R_{work} / R_{free}	0.18/0.223
No. atoms	
Protein	2061
Water	153
B-factors	
Protein (main chain/side chain)	21.1/23.9
Water	34.4
R.m.s. deviations	
Bond lengths (Å)	0.016
Bond angles (°)	1.65

[a] Values in parentheses are for highest-resolution shell.

Supplementary Figures

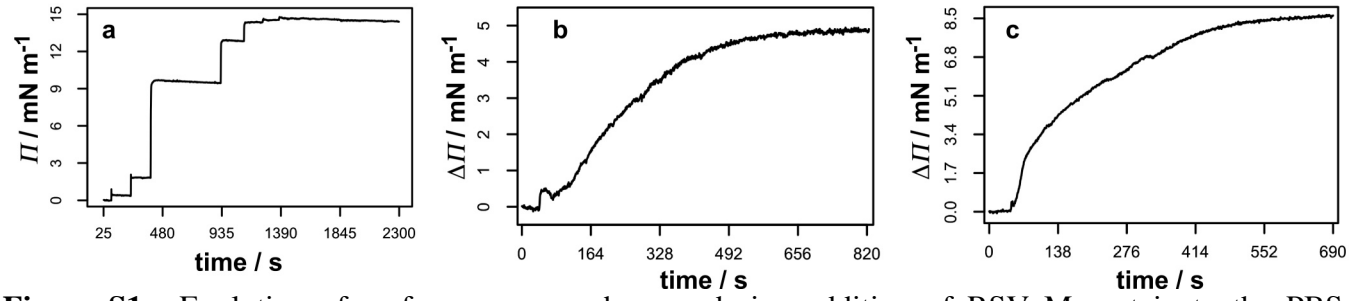

Figure S1. Evolution of surface pressure changes during addition of RSV M protein to the PBS subphase at pH 7.4, 20 °C.

(*a*) In the absence of lipid, c_P / nM = 0.7, 1.4, 3.6, 7.2, 180, 361, 722; (*b*) following addition of RSV M (c_P = 13 nM) to the subphase below an equilibrated DPPC film (c_L = 0.25 μM) at a constant area of 70 Å^2 per lipid molecule; (*c*) following addition of RSV M (c_P = 1.2 nM) to the subphase below an equilibrated DPPE/DOPC film (c_L = 0.12 μM) at a constant area of 146 Å^2 per lipid molecule.

It is apparent from Figure S1, *a-c* that 15-20 min was sufficient to enable surface equilibration {in addition to surface equilibration, each film was partially compressed and expanded 3 times before isotherm data were collected}. Figure S1 *a* presents the raw data used for Figure 1 of the main paper. Figure S1, *b* and *c* show that the protein is able to penetrate densely-packed PC monolayers at physiologically-relevant packing densities.

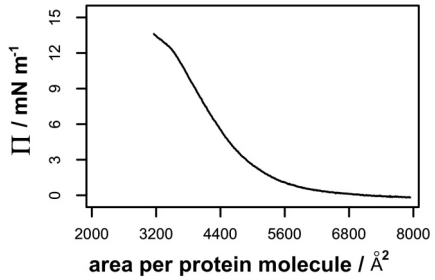

Figure S2. Isotherm for RSV M protein in the absence of lipid on a PBS subphase at pH 7.4, 20 °C, c_P = 3.1 nM.

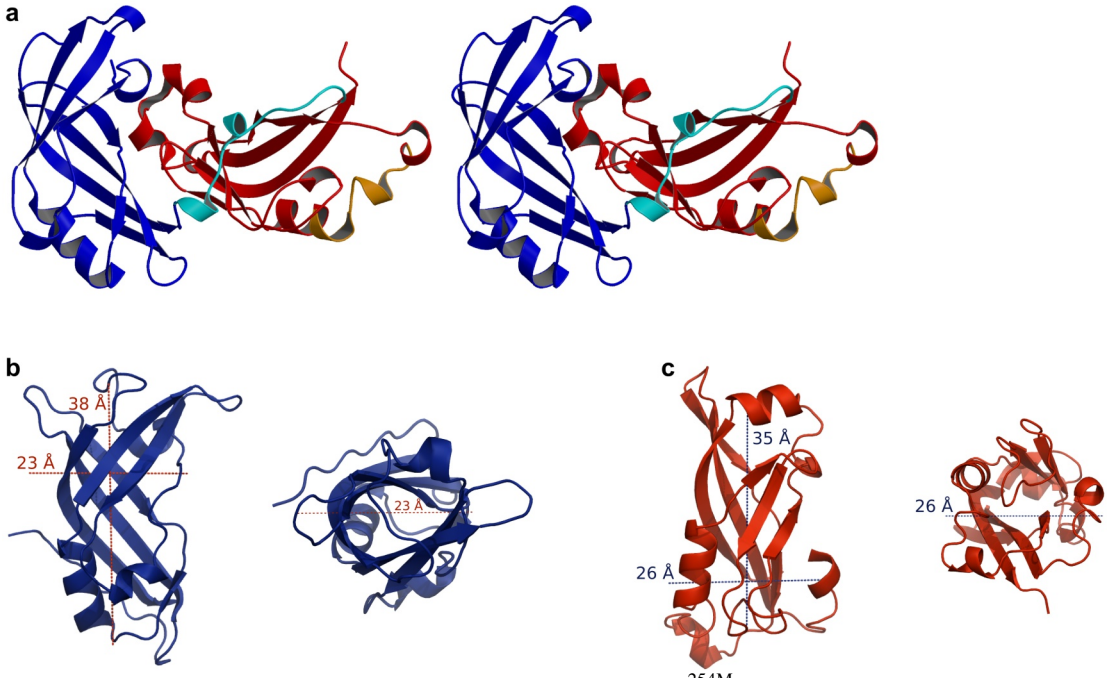

Figure S3. Structure of native RSV M protein (M^{254M}). (*a*) Divergent (wall eyed) stereoview of the protein colored according to domain, with the linker shown in cyan, the N-terminal domain in blue and the C-terminal domain in red. (*b*) orthogonal views of the N-terminal domain, showing key dimensions. (*c*) orthogonal views of the C-terminal domain, showing key dimensions.

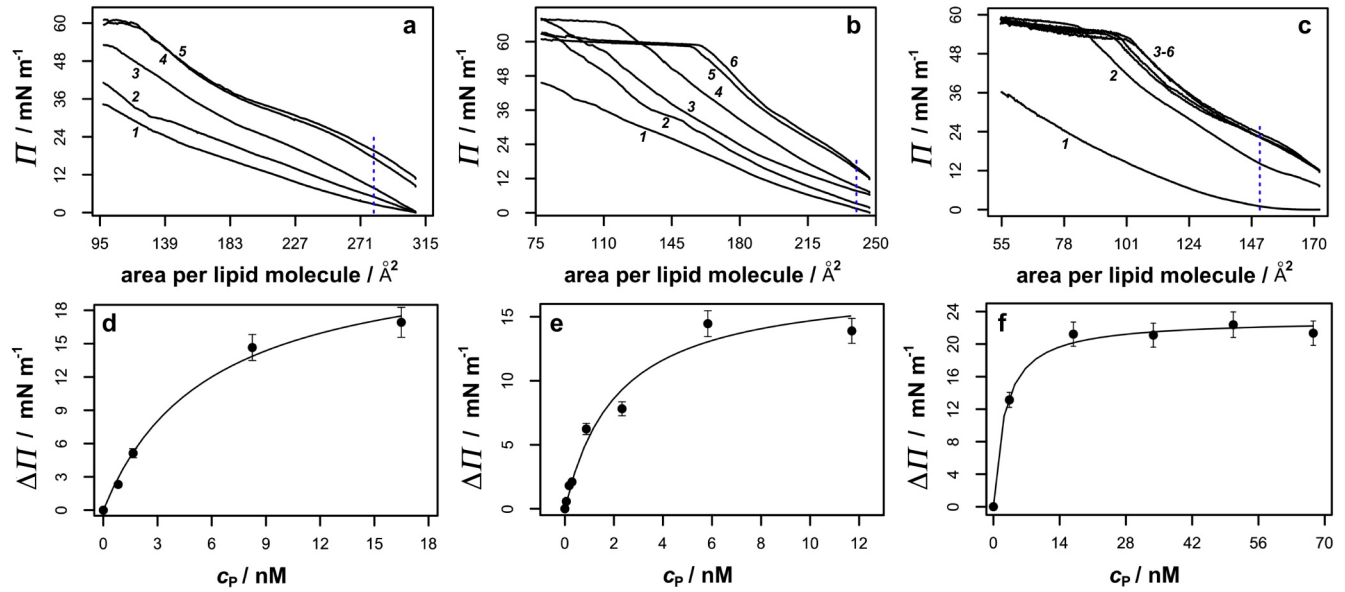

Figure S4. Pressure-area (Π-A) and adsorption isotherms for lipid monolayers spread over a PBS subphase containing RSV M protein at 20 °C, pH 7.4.
(*a*) DOPC, c_L = 165 nM; c_P / nM = 0 (1), 0.8 (2), 1.6 (3), 8.2 (4), 16.5 (5). (*b*) DOPC/DPPC/chol (1:1:1), c_L = 117 nM; c_P / nM = 0 (1), 0.2 (2), 0.9 (3), 2.3 (4), 5.8 (5), 11.7 (6). (*c*) DPPE/DOPC (1:4), c_L = 169 nM; c_P / nM = 0 (1), 3.4 (2), 16.9 (3), 33.8 (4), 50.7 (5), 67.6 (6).
(*d*)-(*f*) Adsorption isotherms corresponding to sections through the Π-A isotherms at constant film area (indicated by a dashed line in (*a*)-(*c*)) as a function of c_P. Areas were selected to avoid the inclusion of Π-A data acquired following film collapse. (*d*) DOPC, A = 280 Å^2 per lipid molecule, ω = 38 Å^2, K = 1.7 × 10^8 M^{-1}. (*e*) DOPC/DPPC/cholesterol (1:1:1), A = 240 Å^2 per lipid molecule, ω = 42.8 Å^2, K = 7.7 × 10^8 M^{-1}. (*f*) DPPE/DOPC (1:4), A = 150 Å^2 per lipid molecule, ω = 39 Å^2, K = 4.2 × 10^8 M^{-1}. Error bars are estimated from the difference in pressure observed in compression and expansion isotherms at each area.

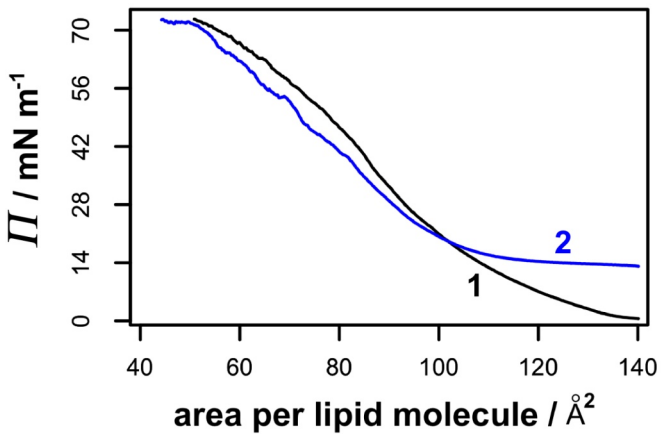

Figure S5. Pressure-area (Π-A) isotherms for films of DOPC/SM/cholesterol (1:1:1), c_L = 207 nM, spread over a PBS subphase (1, black line) or a PBS subphase containing RSV M protein at 20 °C, pH 7.4, c_P = 5.9 nm (2, blue line).

These isotherms were obtained in a similar maner to Figure 2 *d* of the main paper, but with a higher lipid concentration, permitting compression of the monolayer to the collapse point.

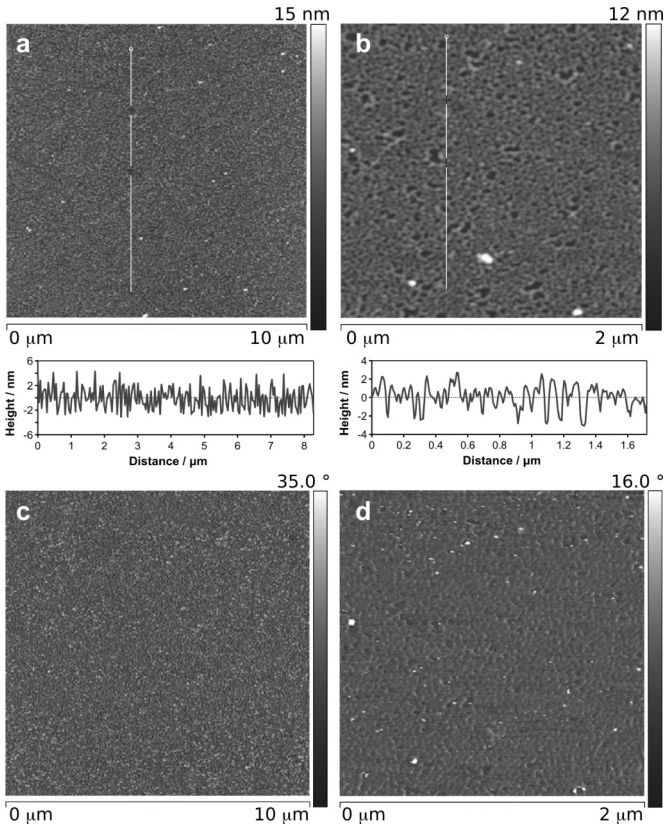

Figure S6. Tapping mode AFM images of surfaces prepared by transfer from monolayers of DOPC/DPPC/cholesterol (1:1:1) and RSV M protein, spread on PBS subphases at 20 °C and pH 7.4, to modified silicon surfaces at Π = 29 mM m^{-1}. Lipid and protein concentrations: $c_L = 7.6 \times 10^{-8}$ M, $c_P = 3.8 \times 10^{-9}$ M. Surfaces correspond to the face of the monolayer in contact with the subphase. Sections through the data are shown below the corresponding image. (*a*), (*b*) Height images reproduced from Figure 4 of the main paper, redisplayed here to allow comparison with their corresponding phase images ((*c*) and (*d*)).

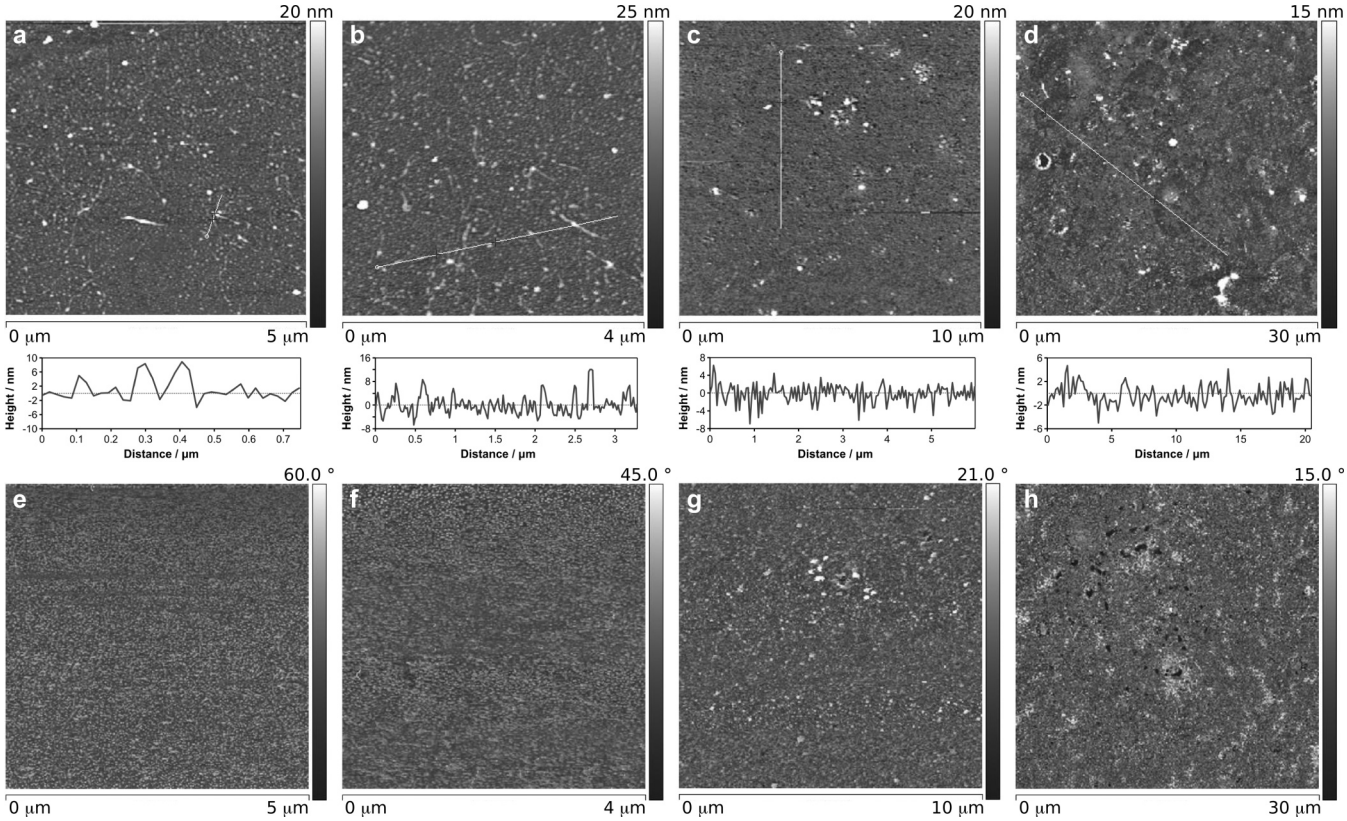

Figure S7. Tapping mode AFM images of surfaces prepared by transfer from monolayers of DOPC/SM/cholesterol (1:1:1) and RSV M protein, spread over PBS subphases at a surface pressure of 17.3 mN m^{-1} at 20 °C and pH 7.4, to modified silicon surfaces.
Lipid and protein concentrations: $c_L = 7.7 \times 10^{-8}$ M, $c_P = 7.7 \times 10^{-10}$ M. Surfaces correspond to the face of the monolayer facing the subphase. Sections through the data are shown below the corresponding image. (*a*)-(*d*) Height scans, with (e)-(h) the corresponding phase images. (*a*), (*b*) Scans from a different surface region to Figure 4 *d* in the main paper. (*c*), (*d*) Images from a repeat experiment (fresh preparation) showing similar features to Figure 4 *d* in the main text.

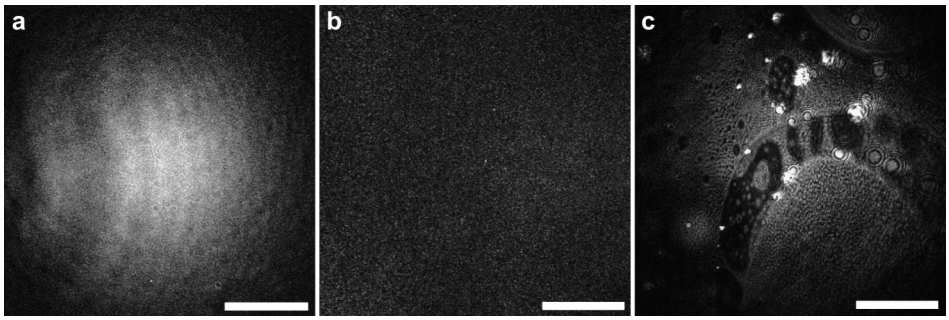

Figure S8. BAM images from compression isotherms of monolayers of DOPC/DPPC/cholesterol (1:1:1) spread over PBS subphases at pH 7.4, 20 °C. Lipid concentration: c_L = 175 nM. Scale bars represent 500 µm in each case. (*a*) A lipid film at Π = 0 mN m^{-1}. (*b*) A lipid film over a subphase containing RSV M (c_P = 4.9 nM) at Π = 14 mN m^{-1}. (c) A lipid film over a subphase containing RSV M (c_P = 4.9 nM) following film collapse (Π = 65 mN m^{-1}). Part (*a*) shows the lack of surface features for the DOPC/DPPC/chol/M film. Part (*b*) demonstrates the lack of surface contrast in the presence of RSV M at the same area per lipid molecule as part (*a*). Part (*c*) demonstrates film collapse.

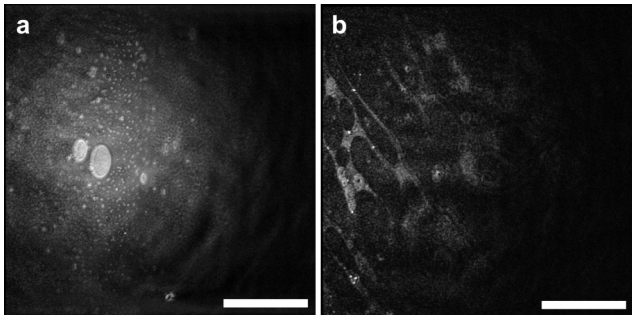

Figure S9. BAM images from isotherms of a DOPC/SM/cholesterol monolayer (1:1:1, c_L = 178 nM) at, spread over a PBS subphase at pH 7.4, 20 °C. Scale bars represent 500 μm in each case. (*a*) Lipid only, Π = 20 mN m^{-1}. (*b*) A mixed lipid/RSV M (c_P = 4.9 nM) film in the plateau region (Π = 12 mN m^{-1}). This shows the formation of small phase-separated domains at higher surface pressure, both in the presence and absence of protein.

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