Supporting Information

Membrane protein distribution in composite polymer-lipid thin films

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Materials - Polymer, Lipid and OmpF Protein
We used amphiphilic poly(2-methyloxadoline)-block-poly(dimethyloxiloxane)-block-poly(2-methyloxadoline) polymer (ABA3) ¹, composition: PMOXA₃₅-PDMS₁₁₀-PMOXA₁₅, mass 10700 g/mol, polydispersity index 1.62. The sulforhodamine B-labelled variant of this polymer, as described previously ², was used for polymer visualization with fluorescence microscopy. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids, Inc. as chloroform solution. Preparation and purification of OmpF were described before ³.

Mixed Langmuir-Blodgett (LB) Films
Polymer-lipid mixtures were prepared in molar ratio 0.3:0.7 from stock solutions with concentrations of 1 mg/mL in chloroform. For solutions containing labelled polymer, 5 % (mol/mol polymer) of ABA3 labelled with sulforhodamine B (SRB) was used.

Glass slides were cleaned with acetone, ethanol and water prior to use. Silicon wafers were cleaned in hot ‘piranha’ solution (concentrated hydrogen peroxide/sulfuric acid, 1:1 v/v) for 10 min, rinsed with bidistilled water, and dried with a nitrogen stream. LB-films were prepared using a Mini-trough (KSV, Helsinki, Finland), surface area 242 cm² with symmetrical lyophilic barriers and a Wilhelmy plate film balance. Before each experiment, the trough was cleaned with chloroform, ethanol, chloroform and water.

To study protein positioning, first a volume of 35 μL polymer-lipid solution was added dropwise to the air-water interface and on silicon wafers, as well as for film thickness determination. The EP3-SW (equipped with a Nd:YAG laser at 532 nm, Nikon 20x long distance objective, and a monochrome CCD camera attached to a real-time frame grabber) was mounted above the Langmuir trough or solid sample stage. Brewster angle microscopy was performed at the angle of incidence of 53°.

ToF-SIMS Measurements
ToF–SIMS measurements (Time of Flight – Secondary Ion Mass Spectrometry) on films transferred to silicon wafers were done with a ToF SIMS 5 spectrometer from ION-TOF (Münster, Germany). We used Bi³⁺ ions, energy of 25 kV, as primary ions for spectra acquisition. All spectra (references and polymer-lipid mixed films) were acquired with high mass resolution, and analyzed as reported previously (see Results and Discussion, page 9853 in ⁴).

In control experiments, we first recorded mass spectra from a clean silicon wafer, an ABA3 film, and a lipid film. We identified the following peaks, which will further help to differentiate between lipid and polymer regions in the mixed film: for ABA3, the characteristic peaks are CN⁻ (m/z 26.00) and CNO⁻ (m/z 85.95), while for DPPC we observe a PO₃⁻ peak at m/z 62.97, a PO₄⁻ peak at m/z 78.92, and a peak corresponding to alkyl chains, C₃₅H₇₂O₃⁻, at m/z 255.25. To determine the spatial distribution of the two components in the film, we performed ToF-SIMS imaging and analysis of the surface coverage and peak intensities in corresponding regions of the film. For example, as described in ⁴, the analysis of surface coverage for a film transferred immediately after 25 mN/m were reached, shows that in star-like structures there is 4.5 % of the overall CN⁻ intensity, 31.2 % of PO₃⁻ intensity, 37.3 % of PO₄⁻ intensity, and 41.5 % of C₃₅H₇₂O₃⁻ intensity (mean values from three measurements at different sample regions). Using these results as a ‘benchmark’, we used a similar procedure to analyze samples after various incubation times.

ToF-SIMS allows for a quantitative analysis of the signal intensity originating from various regions of the sample, resulting in the per-cent of lipid (polymer) signal originating from the domains or the continuous phase. It should be noted that these

AFM Imaging
Polymer-lipid LB films were imaged in contact mode (Nano-Wizard AFM, JPK Instruments AG, D) at room temperature in air with triangular pyramidal probe SiN cantilevers (Olympus Co, J). Images were analysed using Gwydion. Images were levelled, high-pass filtered, and single false lines were interpolated. Cross-sections were measured on straight lines in x-direction, parallel to initial probe movement.

Fig. S1: (A) AFM image of a lipid feature (A); 10x10 points force map of the same area (B). Light pixels represent stiff areas, dark pixels represent soft areas.
results may be affected by the ‘matrix effect’, which means that different energies are required for a molecule to fragment under the ion beam, depending on its surrounding. In this system, DPPC is surrounded by ABA3 in the continuous phase, and by other DPPC molecules in the domains. The calibration of the matrix effect for the ABA3-lipid phase is not possible due to the fact that we are unable to produce homogeneous films with known compositions that would not form domains. In the domains, however, the matrix would be similar (lipid only), so even if the absolute numbers bear an error, it should be in the same range and a qualitative comparison should be feasible.

Fluorescence Microscopy
Images of LB films transferred to glass slides were taken on a Leica DM-RP microscope and a confocal laser scanning microscope (Confocor 2, Carl Zeiss, Germany). Images were contrast-normalized in terms of their greyscale using GIMP (the darkest grey value was assigned black, and the lightest grey value was assigned white). Images with Alexa-Fluor R-488 (labelled protein) were then coloured green and images with SRB (labelled polymer) were coloured red with ImageJ. The corresponding red and green-coloured images were then merged.

Protein Labelling
Prior to labelling, a UV-Vis spectrum was measured of the initial solution of OmpF (in-house purified) in PBS* buffer (16 mM KH₂PO₄, 84 mM K₂HPO₄, 0.1 % n-octylpolyoxyethylene (octyl-POE, w/v), pH 7.5).

OmpF was labelled with Alexa-Fluor R-488, a succinimidyl ester that links specifically to primary amines (in arginine, asparagine, glutamine, and lysine), most of which are located outside of the membrane-embedded hydrophobic region of the protein.

For labelling, 50 μL of 1 M sodium bicarbonate solution were added to 500 μL OmpF solution in PBS*. 1 mg Alexa-Fluor R-488 (Invitrogen, Life Technologies Ltd, UK) reactive dye was dissolved in 100 μL DMSO. 50 μL of the dye solution were added dropwise to the protein solution and the mixture was incubated at room temperature for 1 h under continuous stirring.

A Slide-A-Lyzer (Thermo Fisher Scientific Inc., USA) dialysis cassette (MWCO = 35 kDa) was equilibrated in 50 mL PBS* for 30 min. After incubation, the protein/dye mixture was transferred into the dialysis cassette and dialyzed against a volume of 1 L of PBS. Dialysis was performed at room temperature for 5 days, while the buffer was exchanged every 24 h. After recovering the protein solution, a UV-Vis Spectrum was measured and the labelled protein was stored at 4°C.

Sufficient labelling was validated by UV-Vis spectroscopy. The UV-Vis spectra of unlabelled and labelled OmpF are shown in Figure S2.

The protein concentration after the labelling reaction can be estimated using (maximum) absorption of the dye at 280 nm. After introducing a dye-specific correction factor $CF = \frac{A_{280}(\text{dye})}{A_{495}(\text{dye})}$, the concentration of protein is given by: $[\text{protein}] = \frac{M_w \cdot \frac{A_{280} - CF \cdot A_{495}}{E_{280} \cdot d}}{\frac{A_{495} \cdot M_w}{[\text{protein}] \cdot E_{\text{dye}}}}$. With the computed extinction coefficient ($E_{280}$) 54210 M⁻¹cm⁻¹, molecular weight of OmpF monomer (Mw) 37.08 kDa, a cuvette path length (d) 1 cm, CF for Alexa-Fluor R-488 of 0.11, and measured values of $A_{280} = 1.38$ and $A_{495} = 4.30$, this gives protein concentration (after labelling) of 0.617 mg/mL. Degree of labelling (DOL) is therefore: $DOL = \frac{A_{495} \cdot M_w}{[\text{protein}] \cdot E_{\text{dye}}}$. With $E_{\text{dye}}$ of 71000 M⁻¹cm⁻¹ this yields an average of 3.6 dye molecules per OmpF monomer (10.9 dye molecules per trimer, assuming complete removal of free dye from solution).

Protein Characterization
To control the structural integrity of OmpF after labelling, circular dichroism (CD) spectra were recorded (Chirascan CD Spectrometer, Applied Photophysics Ltd, UK), in units of ellipticity (mdeg) from 180 to 260 nm with a step size of 0.5 nm and signal averaging time of 2 s per point at room temperature. All samples were measured in a quartz cuvette (1 mm path length), previously washed with acetone, ethanol and nanopure water and dried with N2.

Baselines for air, water, PBS*, and the final dialysis buffer were recorded three times. Next, ten CD spectra of unlabelled and ten of labelled OmpF were recorded. Baselines and protein spectra were averaged and smoothed with a Savitzky-Golay filter (3rd order polynomial, smoothing window of 20 points). The smoothed baselines of the respective buffers were subtracted from the smoothed spectra of the proteins.
The spectra of unlabelled and labelled OmpF are shown in Figure S3. Both spectra are virtually identical and exhibit the characteristic pattern of a β-barrel protein with a single minimum at 217 nm. The spectrum of labelled OmpF is only minimally shifted towards positive values and slightly compressed in direction of the y-axis. The latter may be due to the accuracy of the protein concentration determination, while the shift might result from the presence of trace amounts of free dye in solution.

The aggregation state of OmpF (monomer vs. trimer) was confirmed by SDS-PAGE, the gel is shown in Figure S4. Samples of unlabelled and labelled OmpF were run in boiled and unboiled form. The boiled (denatured) samples show at approximately 37 kDa, which matches the mass of OmpF monomers. Unboiled samples run between 80 and 90 kDa. This is slightly below the expected mass of the trimer, however, the phenomenon of running at a mass lower than the theoretical value, is known for β-barrel proteins, due to interaction of the protein’s hydrophobic part with SDS, where SDS does not denature the protein but participates in its solubilization. Thus, this gel suggests an intact trimeric arrangement for both, labelled as well as unlabeled OmpF.

**Fig. S3**: CD spectra of unlabelled (blue) and labelled OmpF (green). The spectra are identical to reference results for fully folded protein.

**Fig. S4**: SDS-PAGE of labelled and unlabelled OmpF. Lanes 1 and 2 are unlabelled OmpF, lanes 3 and 4: labelled OmpF. Samples in lanes 1 and 3 were heated to 100°C for 30 min prior to loading, while lanes 2 and 4 were untreated.