Electronic Supplementary Information

Phase separation in pore-spanning membranes induced by differences in surface adhesion

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Content

1. Fatty acid distribution of sphingomyelin from porcine brain (SM_{porc})[1]

SMs	SM $C_{16:0}$	SM $C_{18:0}$	SM $C_{20:0}$	SM $C_{22:0}$	SM $C_{24:0}$	SM $C_{24:1}$	Unknown
%	2	49	5	8	6	20	10

2. Analysis of fluorescence micrographs of PSMs as a function of temperature

Each fluorescence micrograph obtained for a given lipid composition and at a defined temperature was analyzed individually to extract the fluorescence intensity ratio of the f-PSM and s-PSM. The procedure was performed using a custom-designed Matlab script. Analysis of the image sequence was done in random order to avoid systematic errors of the user. The fluorescence micrograph analysis is schematically depicted in Fig. S1.

Two fluorescence images are obtained for each condition, one showing the BODIPY-Chol fluorescence intensity, the other one the TexRed-DHPE fluorescence intensity. To create the pore grid with pores of radius $r = 0.6$ µm (see Fig. S3), both fluorescence channels were merged and smoothed with a median filter. Pore localization in the merged fluorescence micrographs was automated as pores were not always identified as a circular object. Unidentified pores were extrapolated using the condition of a hexagonal pore arrangement and equidistant spacing between the pores. As a control of the algorithm, the result was checked by visual inspection. If the calculated pore mask did not fit the merged image (bad alignment), some

pore centers had to be set manually, while the rest was extrapolated. The well aligned pore mask with defined pore centers was used to define the theoretical areas of the f-PSMs and s-PSMs. To account for the ambiguity of the fluorescence intensity at the edges between f-PSM and s-PSM, the radius to read out the f-PSM region was set to 0.54 µm (0.06 µm smaller than the pore grid) and the radius to read out the s-PSM region was set to 0.66 µm (0.06 µm larger than the pore grid). Membrane covered regions (f-PSMs and s-PSMs) were defined by threshold analysis. This was achieved by an intensity histogram of the f-PSM region and the s-PSM region, respectively that was created to determine the threshold settings. Each pixel with an intensity larger than the threshold was assigned to membrane, whereas each pixel with an intensity below the threshold was defined as background. Generation of the f-PSM mask was shape-constrained, as pores can only be completely covered by membrane or not. This condition was realized with a filling parameter. If the number of pixels, which is larger than the threshold, was greater than the filling parameter, the whole pore was included in the f-PSM mask, and if not, the whole pore was included in the background mask.

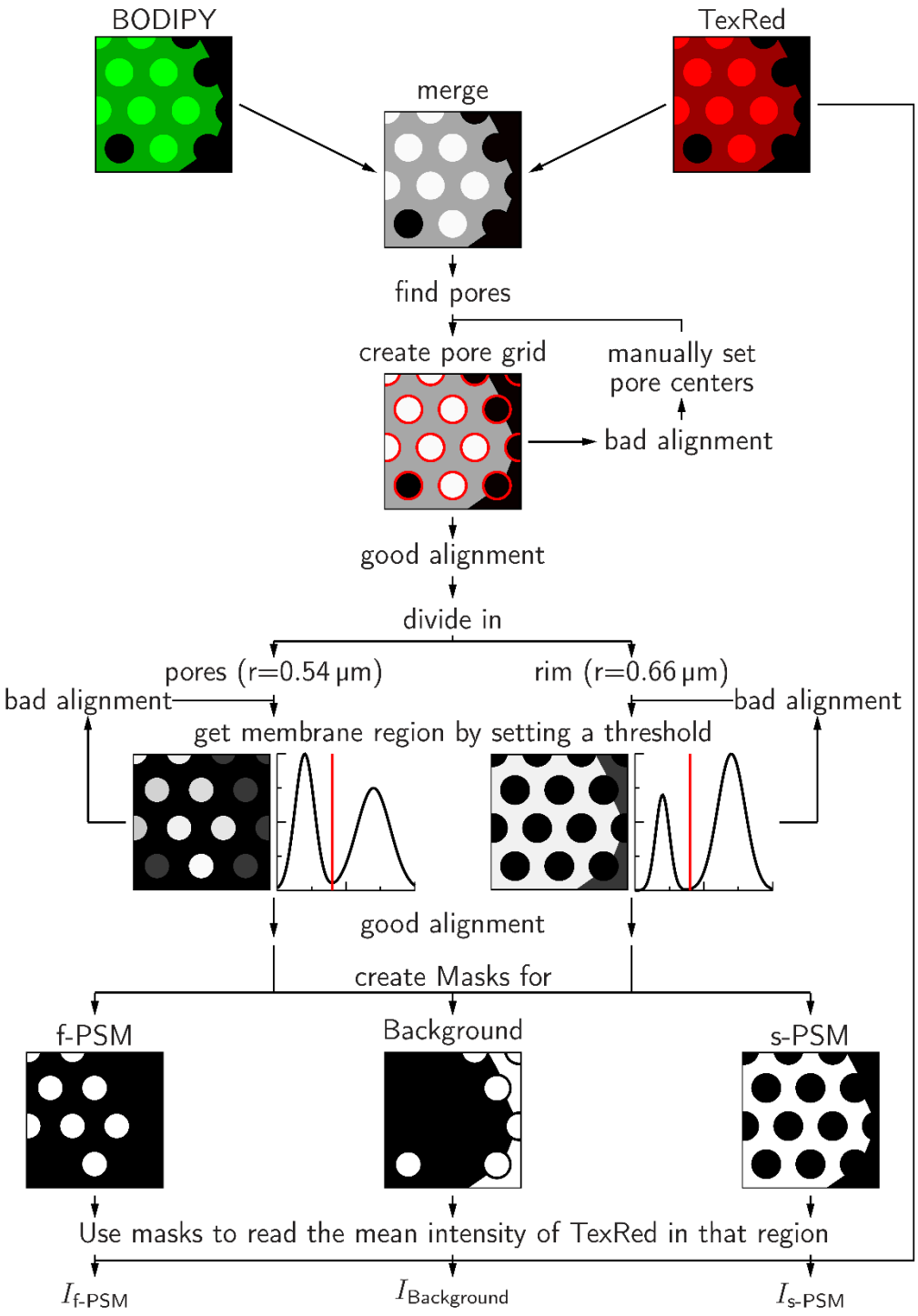

Fig. S1. Process chart for image intensity calculation. The two channels of the fluorescence images (TexRed-DHPE, red and BODIPY-Chol, green) were merged to create the pore grid. The pore and rim regions were extracted and separately analyzed to get the masks for the f-PSM and s-PSM areas. Non-membrane covered regions were identified and assigned as background. These masks were utilized to read out the mean fluorescence intensities of the specific regions ($I_{\text{f-PSM}}$, $I_{\text{Background}}$ and $I_{\text{s-PSM}}$) from the TexRed-DHPE fluorescence images.

The mean intensity (I) of the TexRed-DHPE channel was read out for the different areas by using these masks (f-PSM, s-PSM, Background). Afterwards, the intensity ratio (eq. S1):

$$\frac{I_{\text{f-PSM}}}{I_{\text{s-PSM}}} = \frac{I_{\text{f-PSM}} - I_{\text{Background}}}{I_{\text{s-PSM}} - I_{\text{Background}}} \qquad \text{(S1)}$$

was calculated from the mean intensities of the TexRed-DHPE channel. The calculated intensity ratios of one series were plotted versus temperature T (Fig. S2). The mixing temperature T_M is defined as the turning point of the sigmoidal curve given by eq. (1) (see main text).

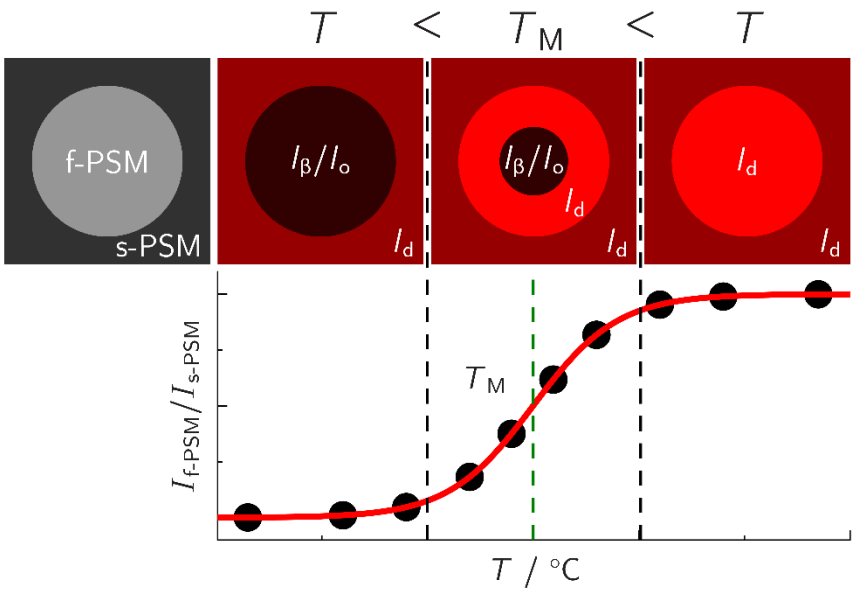

Fig. S2. Schematic illustration of the procedure to extract the mixing temperature T_M from a T-dependent measurement of PSMs. The intensity ratio $I_{f\text{-}PSM}/I_{s\text{-}PSM}$ is plotted against T. The turning point is T_M, which is highlighted by the green dotted line. The intensities of the PSMs at three different temperatures as a function of the phase state of the membrane is illustrated above.

3. Scanning electron micrograph (SEM) of a porous substrate

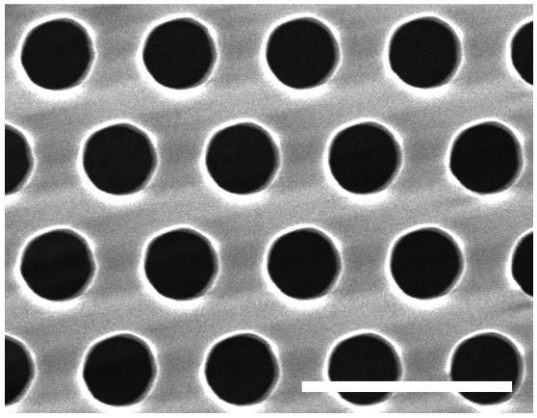

Fig. S3. SEM image of a porous substrate with a pore radius of 0.6 µm. Scale bar: 3 µm. The surface porosity p as determined by taking the hexagonal arrangement of the pores with r = 0.6 µm and an interpore distance of l = 1.72 µm into account reads 44 %. With a threshold analysis of the SEM images, we obtain p = 39 %.

4. Fluorescence images of pure DOPC membranes on $SiO_{x=1\text{-}2}$ functionalized porous surfaces

Fig. S4 shows PSMs composed of pure DOPC to demonstrate the fluorescence intensity difference of both fluorophores (BODIPY-Chol, TexRed-DHPE) on the f-PSMs and s-PSMs independent of membrane phase separation. At both temperatures (T = 25 °C and T = 55 °C) a reduction in fluorescence intensity is observed in the s-PSMs compared to the f-PSMs as a

result of a partial quenching of the fluorophores close to the $SiO_{x=1-2}$ surface. This difference in fluorescence intensity is observed at T = 25 °C as well as at T = 55 °C.

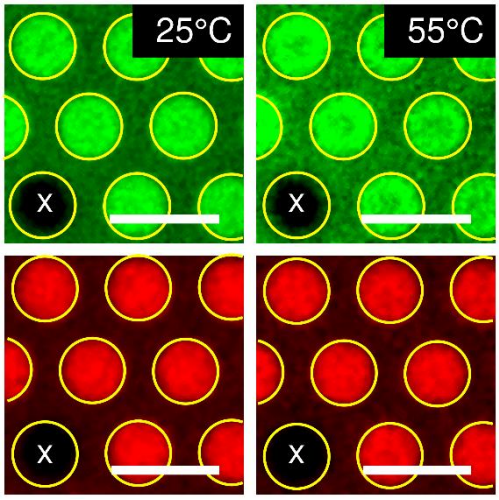

Fig. S4. Fluorescence micrographs of PSMs composed of pure DOPC on $SiO_{x=1-2}$ functionalized porous substrates at T = 25 °C and T = 55 °C. The DOPC membrane is either labeled with 0.5 mol% BODIPY-Chol (top row, green) or 0.5 mol% TexRed-DHPE (bottom row, red). The underlying pores are encircled in yellow. An uncovered pore is marked by an x. Scale bars: 2 µm.

5. Overlay of TexRed-DHPE and BODIPY-Chol fluorescence to identify the gel (l_β) phase in phase-separated PSMs

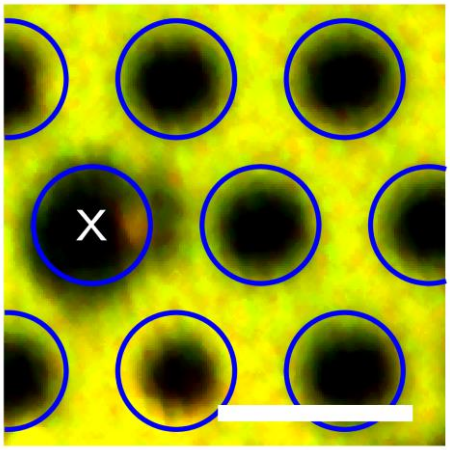

Fig. S5. Overlay of the BODIPY-Chol and TexRed-DHPE fluorescence micrographs of PSMs composed of DOPC/SM_{porc}/Chol (50:50:0) (compare Fig. 1B) at 25 °C. The dark area is assigned to the gel phase l_β, the yellow area depicts the l_d-phase. The underlying pores are encircled in blue. Scale bar: 2 µm.

6. Analysis of hysteresis

To obtain information whether the membrane system is in equilibrium during the heating-cooling cycles under the chosen conditions, we recorded heating-cooling cycles on one membrane patch (Fig. S6). No significant hysteresis was observed.

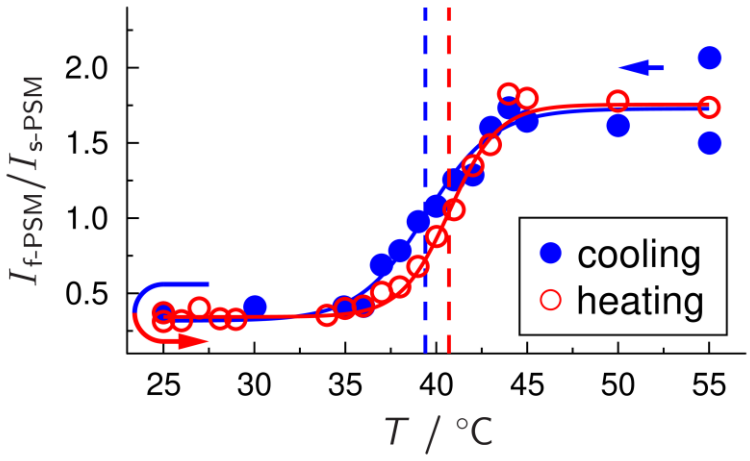

Fig. S6. Relative fluorescence intensity of a PSM composed of DOPC/SM_{porc}/Chol (46:46:8) as a function of temperature. The membrane patch was cooled down and heated up again. Only a small hysteresis with a shift in T_M < 1°C (indicated by the dotted lines) is observed.

7. Overlay of TexRed-DHPE and BODIPY-Chol fluorescence during temperature scan

To visualize the different phases of a DOPC/SM_{porc}/Chol (46:46:8) membrane, overlays of the TexRed-DHPE and BODIPY-Chol fluorescence images are shown (Fig. S7). Both fluorophores are homogeneously distributed above T_M (T = 55 °C). Below T_M (T = 25 °C) the PSM is phase-separated into the l_β-phase and l_d-phase. The f-PSM is in the l_β-phase and the s-PSM in the l_d-phase. At 40 °C near T_M, l_β-domains are still discernible within the f-PSMs, however at the edges, BODIPY-Chol fluorescence intensity can be observed. We speculate that this is a transition region, where the l_β-phase starts transforming into the l_d-phase.

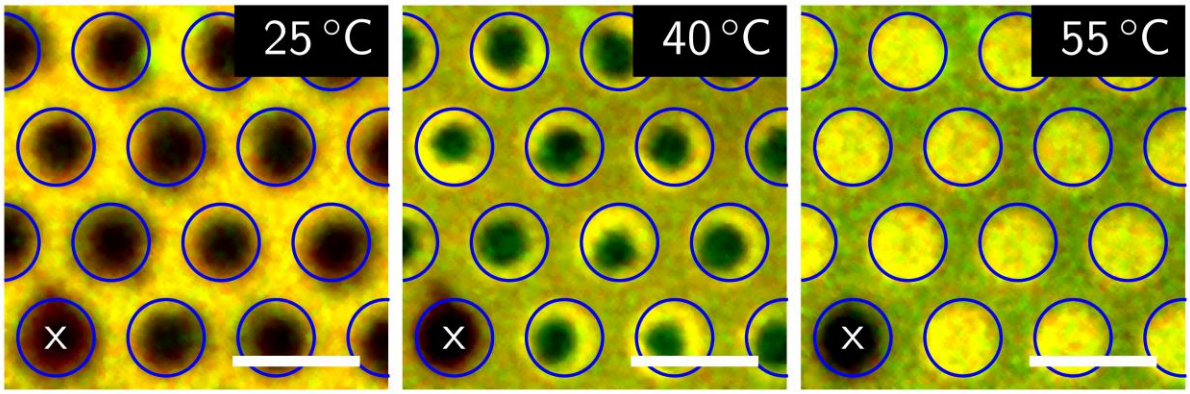

Fig. S7. Overlay of the BODIPY-Chol and TexRed-DHPE fluorescence micrographs of PSMs composed of DOPC/SM_{porc}/Chol (46:46:8) (compare Fig. 2) at 25, 40 and 55 °C. The dark area is assigned to the gel phase l_β, the yellow area depicts the l_d-phase. At 40 °C the f-PSM is separated into the l_d-phase and an intermediate phase between l_o/l_β indicated as some green fluorescence intensity is observed at the domain borders. Scale bars: 2 µm.

8. Determination of the membrane tension as a function of lipid composition

PSMs were prepared according to the procedure described in the Materials and Methods section. GUVs either composed of DOPC doped with TexRed or SM_{porc}/Chol (60:40) doped with BODIPY-Chol were spread on $SiO_{x=1-2}$ functionalized porous substrates. Membrane patches were visualized by the corresponding fluorescent markers and the positions for the AFM indentation experiments were defined using a BIOMAT™ shuttle stage (Bruker Nano GmbH

JPK BioAFM Business, Berlin, Germany). Force distance curves were acquired in the pore center using a NanoWizard IV (Bruker Nano GmbH JPK BioAFM Business, Berlin, Germany) with BL-AC40TS-C2 cantilevers (Olympus, Tokio, Japan). Lateral membrane tensions σ were obtained from the force distance curves according to the procedure described by Nehls et al.[2]

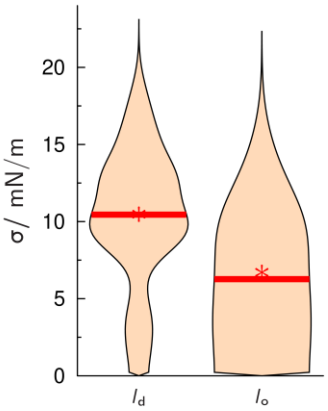

Fig. S8. Violin plots of the membrane tension obtained from f-PSMs on $SiO_{x=1-2}$ functionalized substrates. The red solid line shows the median value and the cross the mean value. GUVs either composed of pure DOPC (l_d) or SM_{porc}/Chol (60:40) (l_o) were spread on the $SiO_{x=1-2}$ functionalized porous substrates. The lateral membrane tension was measured by AFM indentation experiments.

9. Comparison of l_o area fractions in GUVs and PSMs

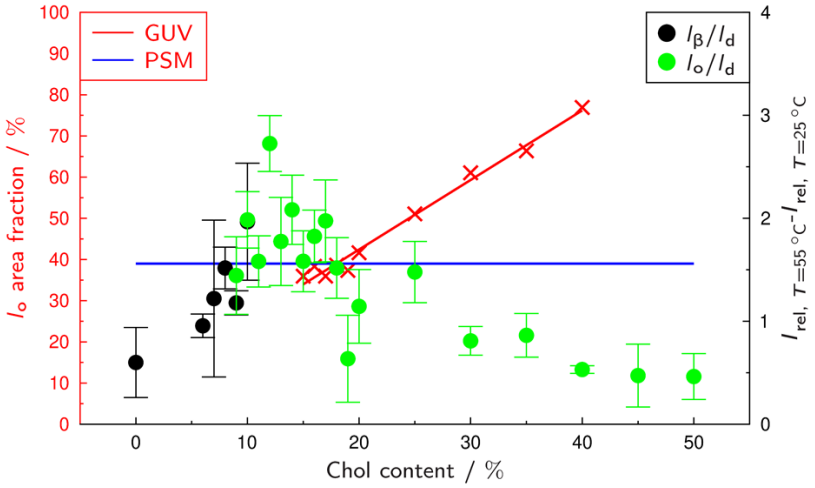

Fig. S9. l_o area fraction of GUVs (red cross) calculated according to the ternary phase diagram of Bezlyepkina et al.[3] (DOPC/SM_{egg}/Chol) together with a linear regression (red solid line) and the l_o area fraction found for PSMs (blue solid line), which is basically determined by the surface porosity as a function of the Chol content n_{Chol} in a lipid membrane (DOPC/SM_{porc}/Chol, 0.5-[n_{Chol}/2]:0.5-[n_{Chol}/2]:n_{Chol}). The maximum fluorescence change ($I_{rel,T=55°C}$-$I_{rel,T=25°C}$) obtained from fitting eq. 1 to the temperature-dependent relative fluorescence intensities as a function of the Chol content is shown for l_β/l_d phase-separated (black dots) and l_o/l_d phase separated (green dots) membranes.

References

1 K. P. Shaw, N. J. Brooks, J. A. Clarke, O. Ces, J. M. Seddon and R. V. Law, *Soft Matter*, 2012, **8**, 1070–1078.

2 S. Nehls and A. Janshoff, *Biophys. J.*, 2017, **113**, 1822–1830.

3 N. Bezlyepkina, R. S. Gracia, P. Shchelokovskyy, R. Lipowsky and R. Dimova, *Biophys. J.*, 2013, **104**, 1456–1464.