Supplementary Material (ESI) for *Soft Matter*

Effect of cholesterol on the rigidity of saturated and unsaturated membranes: fluctuation and electrodeformation analysis of giant vesicles

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Supplementary Information

1. Proteoliposome preparation and lipid extraction from red blood cells

Fresh human red blood cells (RBC) (courtesy of Nina Geldmacher from the Max Planck Institute for Infection Biology, Berlin) were purified according to the method described in detail in [1]. The RBC were washed 4 times in cold isotonic buffer (145 mM NaCl, 5 mM KCl, 5 mM Hepes, pH 7.4 at 4 °C) and centrifuged 10 min at 2000 × *g* at 4 °C. Then, the cells were lysed by shaking 10 min on ice in hypotonic solution (15 mM KCl, 0.01 mM EDTA, 1 mM EGTA, 5 mM Hepes, pH 6.0). EDTA, EGTA and Hepes were purchased from Sigma-Aldrich, Germany. The ghosts were washed once in hypotonic solution without EGTA and twice in hypotonic solution without EGTA but with 2 mM Mg^{2+} and centrifuged for 10 min at 4°C and 12000 × *g*. To remove peripherally associated proteins (and remaining hemoglobin, which is known to contaminate lipid extracts [2]) from the membrane of the open RBC ghosts, they were shaken for 30 min on ice in 10 mM NaOH and centrifuged. The membranes were subjected to protein digestion with proteinase K (final concentration of 3 mg/ml, over night at room temperature), which reduces remaining proteins within the bilayer to their transmembrane helices [1]. Digestion was stopped by adding Pefabloc (5 mM final concentration) and incubation for 30 min. Five washing steps removed all remaining proteinase K and its inhibitor. The last wash was done with 10 mM glucose. Proteoliposomes were dried on conductive glasses and used for vesicles formation as described in the main text. Proteoliposomes contain about 20 area % transmembrane proteins [1].

For preparation of lipid extracts not containing transmembrane peptides, the lipids were extracted from the proteoliposomes using the method of Bligh and Dyer [3]. Briefly, the centrifuged pellet was diluted with bidistilled water to a volume of 1.2 ml, followed by stepwise addition and vortexing of the following solutions: 1.5 ml chloroform, 3 ml methanol, 1.5 ml chloroform, 1.5 ml bidistilled water. After 5 min centrifugation of the obtained solution at 2000 × *g*, the chloroform-rich phase was separated, stored at -80°C and used for vesicle preparation.

2. Contour recognition

We used a home-developed program for recognizing the vesicle contour from the acquired images. The program uses libtiff [4] to read the images and Qt4 [5] to display and treat them. The rest is done in C/C++ and compiled with GNU tools for either Linux or Windows using mingw [6] and DevCpp [7]. For the mathematical treatment Numerical Recipes in C was used [8]. The program reads the images, between 2000 and 8000, and displays them. The contour finding algorithm is split in two parts, first the contour is grossly found, i.e. with pixel resolution, and then it is refined by fitting intensity decays, achieving a resolution better than one pixel.

The first part closely follows the approach in [9]. Initially, the search of the contour is restricted to a circular shell (or an elongated circle, a stadium shape), defined by two concentric circles with different radius. The user chooses these radii considering that the contour of the vesicle in all images is contained within the shell. The intensity profiles in the radial direction for *N* different angles in this shell are extracted from the image by linear interpolation of the pixel value. Gaussian interpolation was also used, but it did not lead to better results while considerably decreasing the speed of the algorithm. For regions of interest with a stadium shape, i.e. in the case of prolate vesicles, two linear and two angular parts were taken. The extracted intensity values from the angular sections define a rectangular trellis from the unwrapping of the shell. Figure S1 schematically illustrates this process.

At this stage, the vesicle contour is represented by an approximately dark line going through the middle of the rectangular trellis, as shown in the last cartoon in Fig. S1. Thus, finding the contour reduces to locating the path joining the two sides of the rectangle with a minimal given cost. In the following, we explain the algorithm used for this purpose.

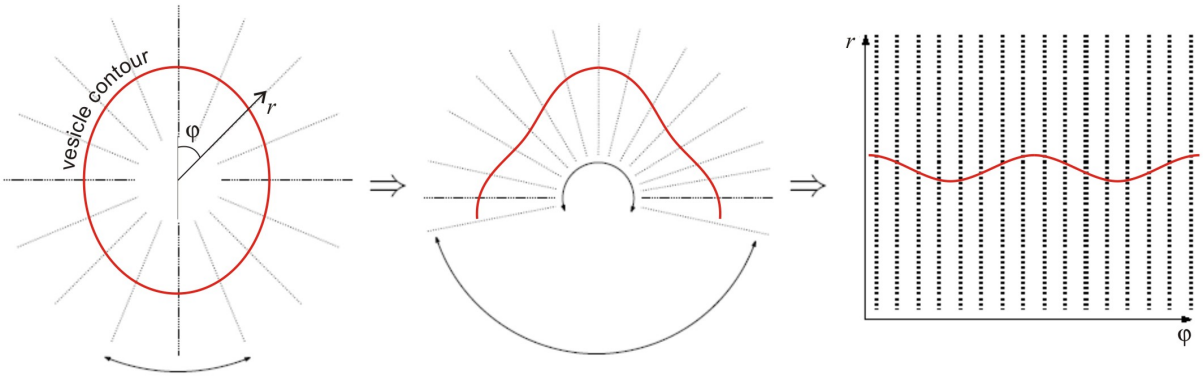

Figure S1. Unwrapping of the trellis to construct a rectangular system for the detection of the vesicle contour. In the first sketch, the contour of an elliptical vesicle is schematically represented by the red curve.

Two scenarios were considered. Both of them have in common that first the gross location of the vesicle contour is found (see next section for a detailed description). After that, we fit the gray value intensity profile around the points from the contour in order to determine it with sub-pixel resolution. The first scenario consists of finding the path with minimal/maximal transversal derivative. Note that a transversal derivative in the trellis corresponds to a radial derivative in the real image (see Fig. S1). Using this approach the steepest part of the intensity profile is found (see Fig. S2).This approach has been previously used in [10], where vesicles filled with sucrose and immersed in glucose were used. The two solutions have different refractive index making the vesicle body look darker than the background. Thus, the intensity profile of a line crossing the contour has a sigmoidal shape. The procedure finds the steepest descending/ascending zone in the line profile and fits it with a straight line. The cross point of this line with the mean gray level of the image give the position of the vesicle contour. The deficiency of this procedure is that perfectly homogeneous illumination is required and no defects (points of significantly different intensity due to, for example, dirt in the sample or in the optics) should be present near the vesicle contour. These problems are overcome in the second scenario, which was primarily used in this work. The contour position is defined as the minimum of the valley in the intensity profile of a line crossing the vesicle contour; see Fig. S2 for one example from a vesicle with no glucose/sucrose contrast enhancement. Note that for vesicles with glucose/sucrose asymmetry, the intensity line profile is not symmetric like in Fig. S2 but has a sigmoidal shape (to illustrate the first approach, we consider a half of the profile, as indicated in the figure). We find the minimum of the intensity profile in gross. Then the two sides of the valley around the minimum are fitted using straight lines and the crossing point defines the location of the vesicle surface; see Fig. S2. The error of this intersection is found from the errors in the fit parameters. In some cases, the fit can be very inaccurate due to defects in the image or impurities. If the location of the intersection (determined with sub-pixel resolution) differs more than 3 pixels from the grossly determined minimum in the line profile, the latter is taken and an error bar of ±1 pixel is given.

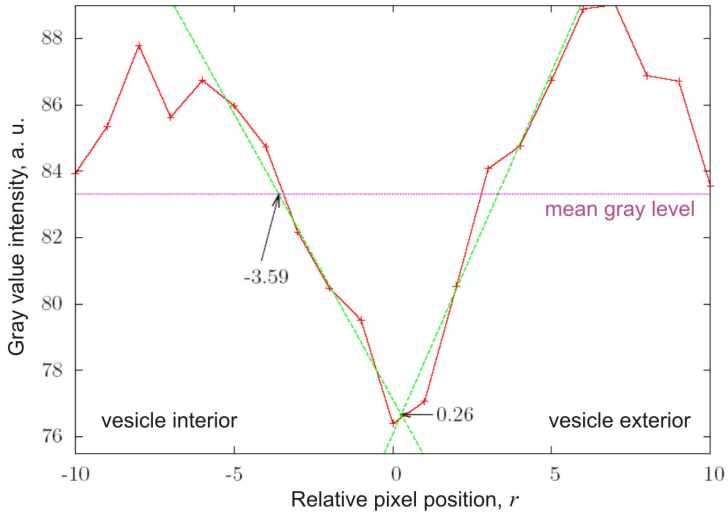

Figure S2. Typical intensity profile of a line crossing the vesicle contour in a phase contrast microscopy image of a vesicle filled with and immersed in the same solution. From the pixel intensity one can locate the valley of the profile (here for convenience placed at $y = 0$) yielding the contour position with pixel resolution. The horizontal line indicates the mean gray level. According to the first protocol, the contour is located at $y = -3.59$ (we have considered only the left half of the intensity profile). The intersection of the steep line in the profile and the horizontal line is used to define the position of the vesicle contour. According to the second protocol (predominantly used in this work), the vesicle contour is located by fitting the two slopes around the minimum with

straight lines (green dashed lines). Their intersection provides a definition of the minimum of the valley, i.e., the location of the vesicle contour in sub-pixel resolution, $y = 0.26$.

Finding the gross contour of a vesicle: In order to find the gross position of the contour we used an efficient dynamical programming algorithm, the so-called Viterbi algorithm. This algorithm uses the fact that if the path with minimal cost from A to C crosses B, the path B to C is also minimal. The algorithm we used is as follows. We consider a trellis with length l and width w. Every point of the trellis is given a weight, I, for instance the value of the derivative in a given direction (the first scenario described above) or the gray value intensity (second scenario). To find the minimum path, a weight function is defined as

$$F_{i,v} = I_{i,v} + \min\left(F_{i-1,v-1},\ F_{i-1,v}, F_{i-1,v+1}\right) \qquad \text{(S1)}$$

where v varies from 1 to w and i varies from 1 to l, and min denotes the minimum value. To fill up the matrix F, one starts from one end of the trellis $i = 1$ (whereby $F_{0,v} = 0$), and evolves step by step till the other end $i = w$. Note that the values of a column $i - 1$ define the values at column i. Once the end of trellis is reached, the minimum $F_{l,v}$ is taken and the minimal path is found by retracing back the choices made for the minimum in eq. (S1). Since we look for a continuous path, without jumps, in the weight definition eq. (S1) we restrict the search to the nearest neighbors only, $v - 1$, $v + 1$. Other options like penalizing curvature have been proposed in literature [9].

This procedure does not necessarily yield a closed vesicle contour, i.e. the last point does not necessarily coincide with the first one. To solve this problem we make use of periodic boundary conditions. The trellis is extended with several points (typically 10 or more), i.e. the beginning of the trellis is copied in the end of it proceeding with the algorithm in eq. (S1). The initial, repeated points, are neglected afterwards, when retracing the path. This procedure proved very successful in closing the contour in about all our images.

The error of the contour is defined as the maximal error of all the points. If this error exceeds a certain threshold, the contour is rejected from the analysis. Furthermore, the contour must be a continuous line; therefore neighboring points must have approximately the same radius. Thus, contours showing large jumps are rejected as well.

Once the contour is found, its center of mass is used to center a shell (two concentric circles) confining the vesicle contour, which is used as a starting search area for the next image. This procedure properly follows the movements of the vesicle if they are not too abrupt and the shell width is large enough. Even vesicles which partially move out of the image borders and come back are followed successfully (the incomplete contours are of course neglected).

3. Dye exclusion from solid domains in vesicles in the three-phase coexistence range

We prepared vesicles composed of DOPC, egg SM and cholesterol with 40:40:20 molar ratio following the method described in the main text. Membranes with this composition at low temperatures like 10°C lie in the region of coexistence of solid (S), liquid ordered (Lo) and liquid disordered (Ld) phases. Thus the vesicles exhibit three types of domains. The membranes also contained small fractions of two dyes (0.3 mol %): perylene and diIC18. diIC18 preferentially partitions in the Ld phase and is excluded from the other domains. Perylene partitions in the Lo and in the Ld phases and is also excluded from the S domains. When observed with confocal microscopy, as shown in Fig. S3, vesicles with three-phase coexistence exhibit Ld domains (red), Lo domains (green) and S domains (black); see Fig. S3. Note that the Ld and Lo domains have smooth boundaries when they are in contact with each other, and rough borders when in contact with the S domains. In two-phase SM:Chol vesicles containing Lo and S domains, perylene should be also excluded from the S domains, and thus can be used as an indicator for their presence. Since we did not observe such exclusion in these vesicles, we conclude that the membranes were either in the liquid ordered state, or that the solid domains were smaller than 1 μm.

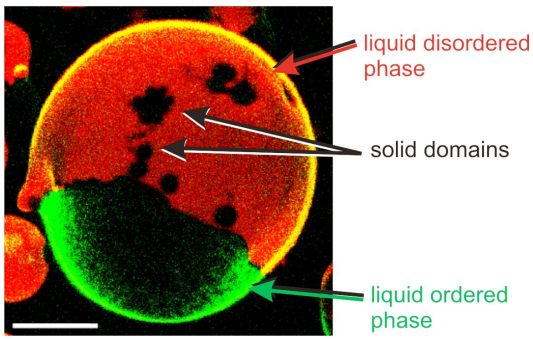

Figure S3. Three phase coexistence in a vesicle composed of DOPC:SM:Chol 40:40:20 at 8.5°C. The scale bar corresponds to 20 μm.

References:

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