Electronic Supplementary Information (ESI)

Utilizing an aqueous-liquid crystal interface to investigate membrane protein interactions and mutation effects of a pore-forming toxin

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Experimental section

Materials:

Required Glass microscopic slides of Fischer's Finest Premium Grade brand were bought from Fischer Scientific in Pittsburgh, Pennsylvania. These glass slides are piranha cleaned by using Sulfuric acid (H_2SO_4), Hydrogen peroxide (H_2O_2, 30% w/v) bought from Merck, and to neutralize it, Sodium chloride (NaCl) was also purchased from Merck. Chloroform (HPLC), 4′-pentyl-4-biphenylcarbonitrile (5CB), dimethyloctadecyl[3-(trimethoxysilyl)propyl]-ammonium chloride (DMOAP) were supplied by Sigma Aldrich. Required lipids, i.e., 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and cholesterol, were bought from Avanti Polar Lipids, Inc (Alabaster, AL). Ethanol was supplied by Jebsen & Jenssen GmbH and Co., Germany (S D fine-chem Limited). 1,2-dioleoyl-*sn*-glycero-3-phosphocholine-N-(Cyanine 5) [Cy5 labelled POPC], 3β-Hydroxy-5,7,22 ergostatriene (Ergoesterol) were ordered from Merck. Alexa Fluor 488 maleimide was used to label LLO, and its mutant was purchased from Thermo Fischer Scientific. Gold grids of 20 μm thickness (50 μm wide, 283 μm grid spacing) were bought from Electron Microscopy Sciences (Fort Washington, PA). We bought Luria Broth (LB media) and Coomassie bright blue dye from Himedia. IPTG (isopropyl-D-thiogalactopyranoside) was obtained from BR Biochem Life Sciences. All buffers were prepared using Milli-Q water, and the required pH was adjusted using a Metrohm 827 lab pH meter at room temperature (~25 °C). The Millipore technology (Bedford, MA) was used to obtain Milli-Q water.

Methods

Piranha Cleaning and Coating of Glass slides:

Glass slides were cleaned with piranha solution (H_2SO_4:H_2O_2 (v/v) = 70:30) in accordance with accepted procedures and reproduced for the reader's convenience.[1,2] Briefly, the freshly prepared piranha solution was added to the glass slides in a glass jar and then placed in a water bath at 80°C for 1 h. After that, the slides were rinsed with ethanol and deionized water. The slides were dried using nitrogen gas purging and maintained in an oven set at 100 °C for at least 3 h. These slides were submerged in a 0.1% (v/v) DMOAP aqueous solution in DI water for 30 min at room temperature to coat them with a hydrophobic medium. After that, washing was done to remove the excess DMOAP before nitrogen drying. These slides were also heated for 3-6 h in the oven to promote cross-linking and the formation of a siloxane bond. The octadecyl chains of the DMOAP and the alkyl tails of 5CB molecules interacted hydrophobically because of the chemical modification of the glass slide's surface with a DMOAP substance, which encouraged LC molecules to align perpendicularly to the substrate.

Preparation of vesicles of PC with varying amounts of cholesterol:

The vesicles are prepared using earlier known method and reproduced here for the reader's convenience.[2] Briefly, to prepare mixed lipid vesicles, different amounts of cholesterol (0%, 15%, and 30% by weight of total lipid) were incorporated in PC vesicles or liposomes. For this, the predetermined amount of lipid was obtained and suspended in chloroform in a round bottom flask. The necessary amount of cholesterol (0, 15 and 30% of cholesterol by weight) was added, and the mixture was then dried for 3 h at room temperature under a vacuum. The dried lipid was rehydrated with PBS buffer after 3 h, and then freeze-thaw cycles were performed to create lipid vesicles. Small unilamellar vesicles were produced during probe sonication of the aqueous lipid dispersion for 30 min. Within 24 h, all the produced lipid solutions were utilized.

Purification of recombinant LLO and its mutant ($LLO^{T515G-L516G}$):

The recombinant LLO and its mutant purification has been done using earlier known method and reproduced here for the reader's convenience.[3] Nucleotide construct of LLO in the pPROEX HTb vector was kindly provided by Dr. Gregor Anderluh, Kemijski inštitut/National Institute of Chemistry, Slovenia. The nucleotide sequence encoding LLO was cloned into the pET14b vector and transformed into *Escherichia coli* Origami B cells (Merck). The culture of the transformed cells was induced with 0.5 mM IPTG (B. R. Biochem) at 30 °C for 3-4 h during the mid-log phase for the overexpression of the protein. The cells were then harvested, lysed, and separated into soluble and insoluble fractions using centrifugation. Ni NTA Agarose chromatography (QIAGEN) was employed to purify His-tagged protein from soluble fractions. The eluted protein was further subjected to cation-exchange chromatography to remove the imidazole and further purify the protein. The eluted protein was visualized using SDS-PAGE/Coomassie staining, and the protein concentration was estimated using absorbance values at 280 nm and the theoretical extinction coefficient.

For the $LLO^{T515G-L516G}$, PCR-based mutagenesis was used to generate the mutation, confirmed with DNA sequencing. Overexpression of the $LLO^{T515G-L516G}$ was achieved by overnight induction of cells at mid-log phase with 0.5 mM IPTG at 20 °C. The protein was purified using the same methodology as described above for LLO.

Circular dichroism (CD) measurements:

A Chirascan spectrophotometer (Applied Photophysics, U.K.) with a scan range of 200–260 nm and a step size of 1 nm was used for the far-UV CD studies. The solution of interest was kept in a quartz cell with a 1 mm path length. Freshly made aqueous solutions of 2 µM LLO and $LLO^{T515G-L516G}$ in 10 mM PBS (pH 7.2) were used. Using the ProData software that came with the CD instrument, the spectra for each sample were averaged over 5 scans and adjusted against the buffer signal.

Preparation of LC-aqueous interface:

The preparation of the LC-aqueous interface has already been described in earlier reports.[2,4] In short, DMOAP-coated glass slides were broken into tiny bits and overlaid with gold grids. After adding roughly 0.2 µl of 5CB to the grids, the extra 5CB was syringed out to create a consistent film of LC. The constructed system was placed in an optical well containing (10 mM, 2 ml) PBS buffer to create the LC-aqueous interface.

Decoration of lipid at LC-aqueous interface:

The LC-aqueous interface was encased in lipid layers composed of the produced lipid vesicles, which were subsequently incubated for 30 min. Following the interval of equilibration, excess lipid in the solution was eliminated by three washes in PBS buffer (10 mM, pH = 7.2). Rinsing was done carefully to avoid disrupting the lipid assembly or the LC film.

Further, the buffer was replaced with various concentrations of the protein, and the optical response of LC was studied under a polarizing optical microscope (POM) to better understand protein interactions with the lipid-rich LC-aqueous interface. To prevent turbulence in the aligned layer of lipid, a variable amount of protein was dispersed slowly and from the optical well's walls.

Optical Characterization of LC films:

In earlier papers, optical characterization has already been mentioned and reproduced here for the reader's convenience.[4] Briefly stated a POM (Zeiss Scope.A1) in transmission mode was used to observe the optical response of LC. Each image was focussed and taken using a Q-imaging digital camera coupled to the POM with an exposure time of 80 ms. The optical well housing the 5CB film

was placed on a revolving platform. The orthogonal orientation of the polarizer and analyzer examined the orientation of 5CB. In each trial, pictures were taken while maintaining a source intensity that was roughly 40% of the brightness.

Quantification of optical responses:

The grayscale intensity of the optical micrographs was used to quantify the LC optical response at different intervals and concentrations. Using ImageJ software, the average grayscale intensity was calculated.[2] To get the mean grayscale intensity, the grayscale intensity was averaged over four grid squares on each micrograph.

Pull-down based binding and oligomerization:

Pull-down based binding and oligomerization have been done using earlier known method and reproduced here for the reader's convenience.[3] For the binding assay, 1 µM of LLO was incubated with 220 µg of lipid vesicles at 4 ºC. The reaction was then subjected to ultracentrifugation at 105000 x g for 30 min. Subsequently, the supernatant was collected, and the pellet was washed twice with 1XPBS buffer. The pellet was then resuspended back to the original volume. An equal amount of supernatant and pellet fraction was analyzed by SDS-PAGE/Coomassie staining. For the oligomerization assay, 1 µM of LLO was incubated with LC/LC-vesicle or vesicle alone at room temperature for 30 min, followed by centrifugation at 16,400 x g for 30 min. The pellet containing oligomeric assemblies was washed twice with 1XPBS and resuspended in 50 µl 1XPBS. Samples were then analyzed using SDS-agarose gel electrophoresis (AGE)/Coomassie staining.

Statistics:

The data are reported as means ± standard error of the mean. A multifactorial repeated measures ANOVA was employed,[5,6] to examine variations in the mean grayscale intensities of LLO and its mutant during interaction with PC containing 30 wt% and 15 wt% cholesterol at the LC-aqueous interface after 30 min of incubation. Subsequently, Tukey's post-hoc analysis was conducted to delve deeper into the findings. Statistical significance was determined at $p < 0.05$.

Labelling of LLO and its mutant ($LLO^{T515G\text{-}L516G}$):

The wild type and mutants were labelled with Alexa Fluor 488-maleimide using the accepted protocol[2] recommended in the manufacturer's kit. Briefly, protein and Alexa Fluor 488-maleimide were mixed slowly in a molar ratio of 10:1 (dye: protein) and incubated at 25 °C for 3 h in the dark. The labelled protein was then subjected to ultrafiltration to remove the free dye and buffer exchange using Amicon ultrafilters with a nominal molecular weight cut-off of 3 kDa. The protein concentration was determined using an absorbance value of 280 nm.

Confocal Fluorescence Microscope Imaging:

We performed confocal microscopic imaging to observe the fluorescently labelled lipid PC and protein localization. To create the samples, a 2.5% Cy5 fluorescently labelled PC lipid combined with 15 wt% cholesterol was incubated with 100 nM Alexa-488 fluorescently labelled LLO and mutant ($LLO^{T515G\text{-}L516G}$). A lens of 63x and hybrid detectors were employed to image the sample. Image acquisition was set to 512×512 pixels with a 400 Hz scanning rate. 10% of the argon laser power was used to excite Cy5 and Alexa-488 fluorescent dyes while keeping laser gain constant at 5%. For the excitation of Alexa-488-labeled LLO and its mutant ($LLO^{T515G\text{-}L516G}$), a 488 nm laser line was used, whereas a 650 nm laser was used for the excitation of Cy5. Stacks of confocal images were captured using an SP8 upright confocal microscope.

Atomic Force Microscopy (AFM) imaging:

For AFM imaging, we followed the procedure outlined in earlier reports.[2,7] Briefly, 100 nM LLO was incubated with a lipid-decorated LC film (composed of PC and 30% cholesterol) for 30 min. After incubation, the glass slide was removed, and approximately 100 µl of PBS buffer (10 mM, pH 7.2) was applied to the grid to collect the sample in contact with the LC film. About 30 µl of the sample was then placed onto a cleaned silicon (Si) wafer and air-dried. Once dried, the sample on the Si wafer was rinsed with 100 µl of milli-Q water and dried under nitrogen flow for 15 min. AFM images were acquired in tapping mode using an Innova Bruker AFM with an antimony-coated silicon tip (8 nm radius), and the images were processed using WSxM software.[8]

Supporting figures:

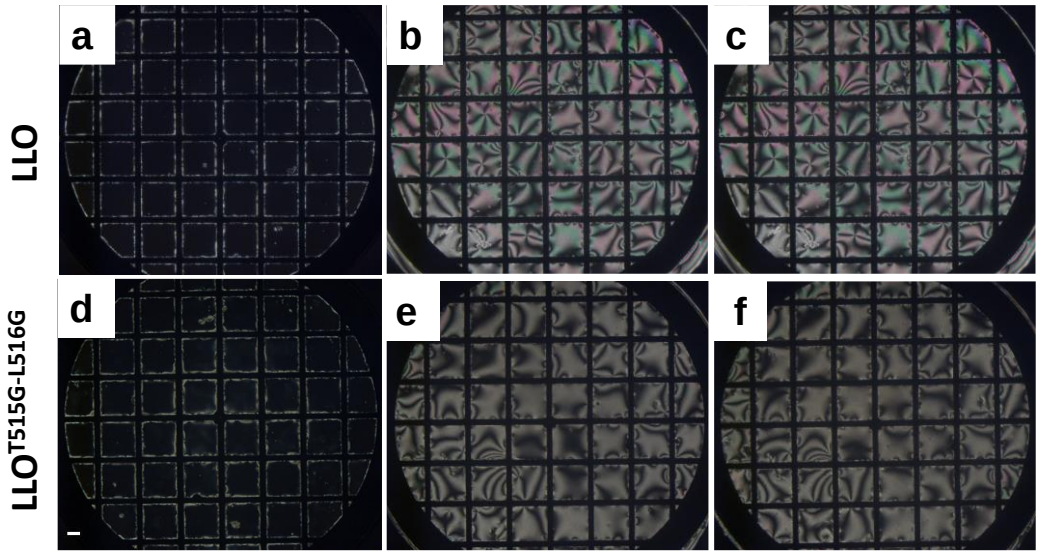

Fig. S1 Optical images in (a, d) depict the LC-air interface under crossed polarizers POM. Photomicrographs in (b, e) displayed the same LC-film view under POM on immersion in PBS buffer containing 200 nM LLO and $LLO^{T515G-L516G}$. The consistency of view in images (c, f) captured after 2 h demonstrates no direct interactions of the protein with LC. Scale bar = 100 μm.

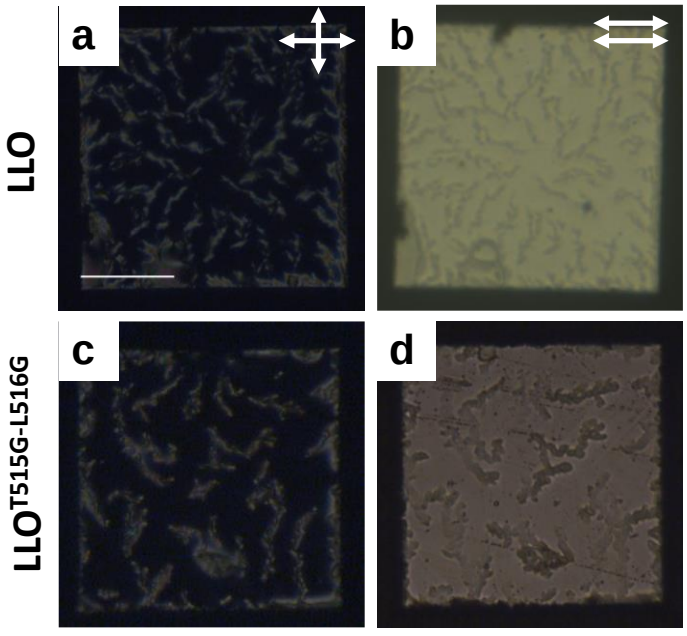

Fig. S2 Polarized optical images in (a, c) depict respective LC's response of 100 nM LLO and $LLO^{T515G-L516G}$ upon incubation with 15 wt% cholesterol in the PC-laden aqueous interface of LC. Optical images (b, d) corresponding to (a, c) displayed similar dendritic patterns after the removal of the analyzer. The arrows on the top left of the image (a) and (b) show the orientations of the polarizer and analyzer during imaging. Scale bar = 100 μm.

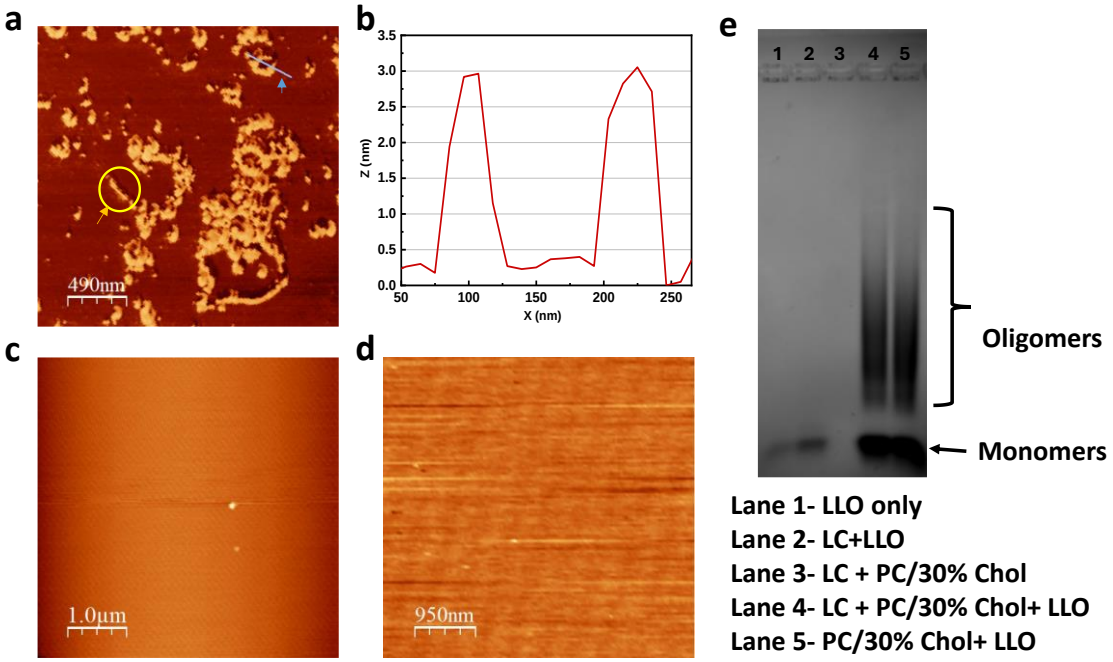

Fig. S3 AFM images (a, c, and d) show the aqueous layer at the LC-aqueous interface. (a) Lipid mixture (PC with 30 wt% cholesterol) after 30 min of incubation with 100 nM LLO, (c) 100 nM LLO without any lipid, and (d) lipid mixture (PC with 30 wt% cholesterol) without LLO. Panel (b) presents the height profile of the arc-like assembly indicated by the blue arrow in the image (a). The yellow circle highlights the formation of filamentous structures. Image (e) shows the pull-down based oligomerization assay of LLO.

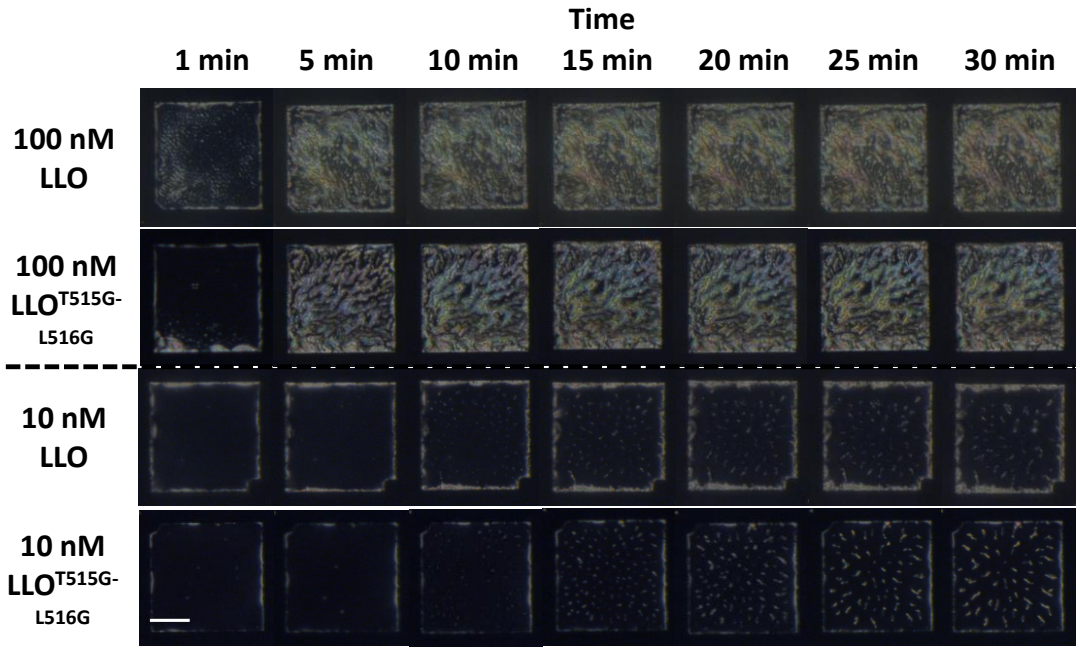

Fig. S4 Time-lapsed images of LC response in the presence of 100 and 10 nM proteins added on LC-aqueous interface laden with PC/30 wt% cholesterol. The images depict the similar activity of both proteins, indicating no difference in the binding of proteins even in the presence of CRM. Scale bar = 100 µm.

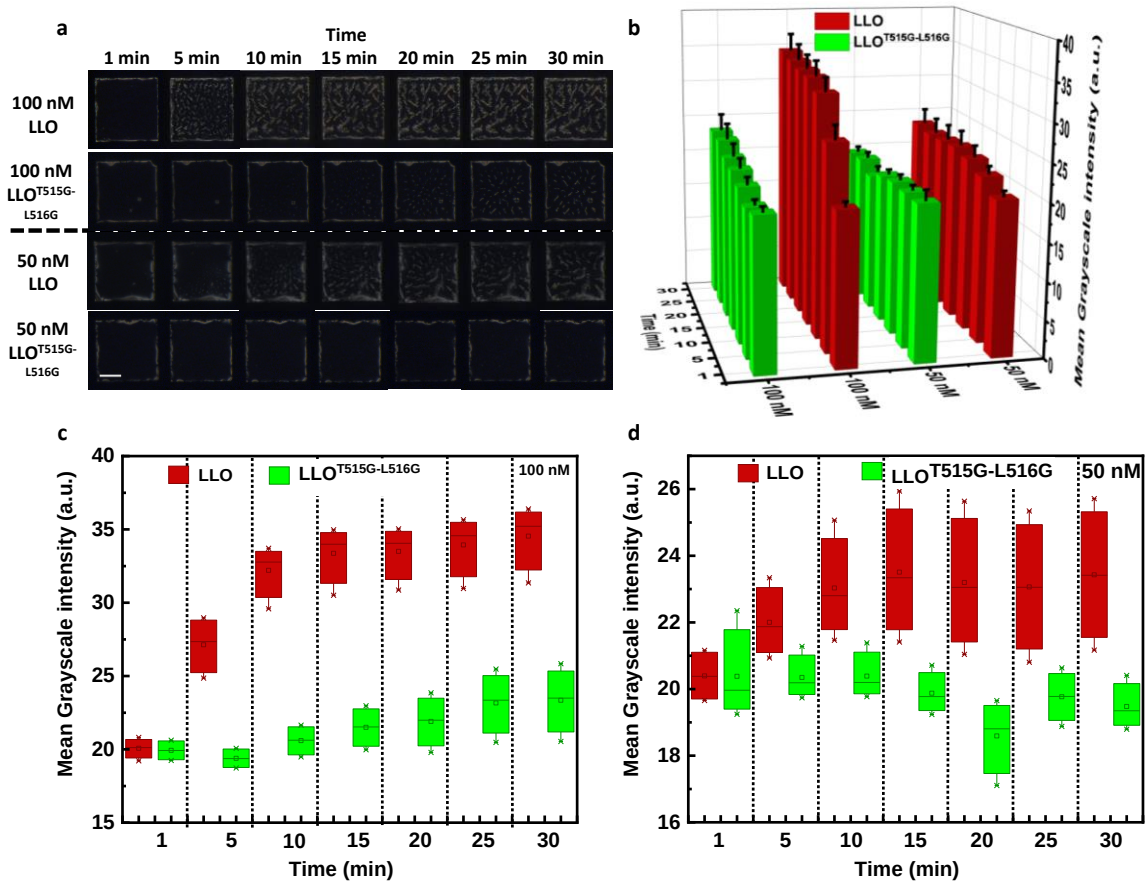

Fig. S5 The optical images in (a) illustrate the time-dependent LC response encased with 15 wt% cholesterol in PC upon contact with 100 and 50 nM proteins (LLO and LLO$^{T515G-L516G}$). The images indicate substantial differences in the activity of both proteins through differential emerging rates of domains. Scale bar = 100 µm. The bar graph in (b) showed the mean grayscale intensities of PC/15 wt% cholesterol-laden LC-aqueous interfaces at different time points in the presence of LLO and LLO$^{T515G-L516G}$. The box plot in (c & d) illustrates the distribution of mean grayscale intensities of PC/15 wt% cholesterol-laden LC-aqueous interface from 1 to 30 min in the presence of (c) 100 nM & (d) 50 nM of LLO and its mutant. One-way ANOVA was used to measure the level of statistical significance. c) p = 0.8 (1 min), $p = 2\times10^{-4}$ (5 min), $p = 2\times10^{-5}$ (10 min), $p = 5\times10^{-5}$ (15 min), $p = 8\times10^{-5}$ (20 min), $p = 3\times10^{-4}$ (25 min) and $p = 3\times10^{-4}$ (30 min). d) p = 0.9 (1 min), $p = 3\times10^{-2}$ (5 min), $p = 1\times10^{-2}$ (10 min), $p = 1\times10^{-2}$ (15 min), $p = 5\times10^{-3}$ (20 min), $p = 1\times10^{-2}$ (25 min) and $p = 8\times10^{-3}$ (30 min).

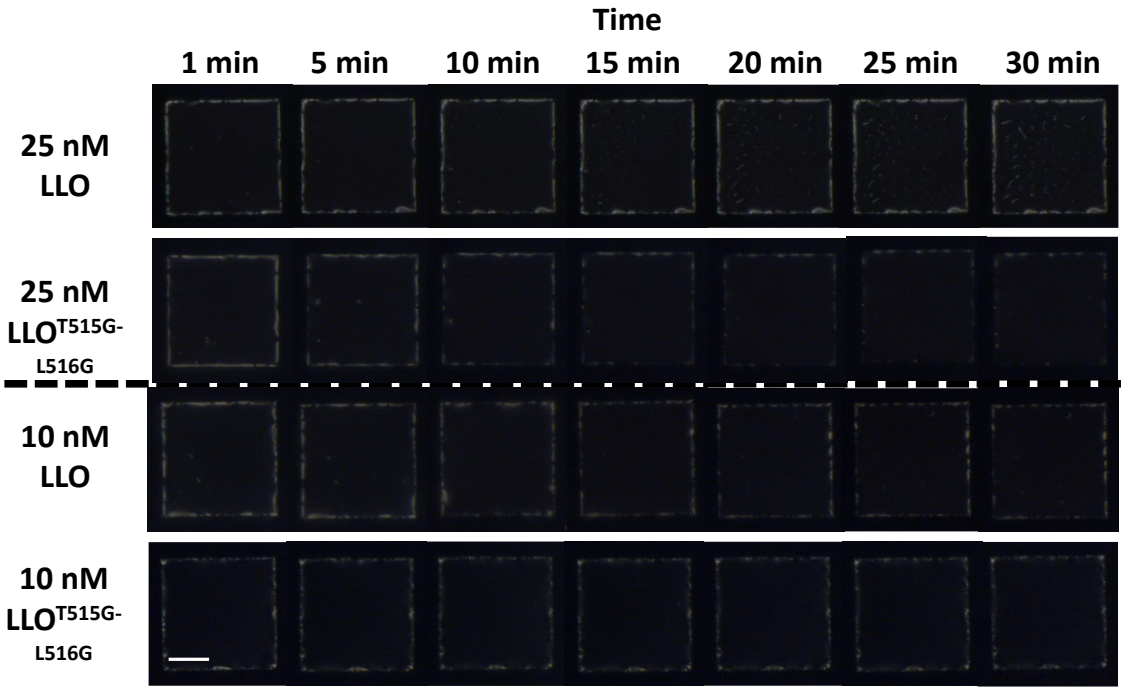

Fig. S6 Time-lapsed polarized optical images of LC response in contact with 25 and 10 nM proteins at 15 wt% cholesterol in PC lipid-laden LC-aqueous interface. The data demonstrates 25 nM as the minimum concentration of LLO required to study LC responses in 15 wt% cholesterol/PC-laden LC. Scale bar = 100 µm.

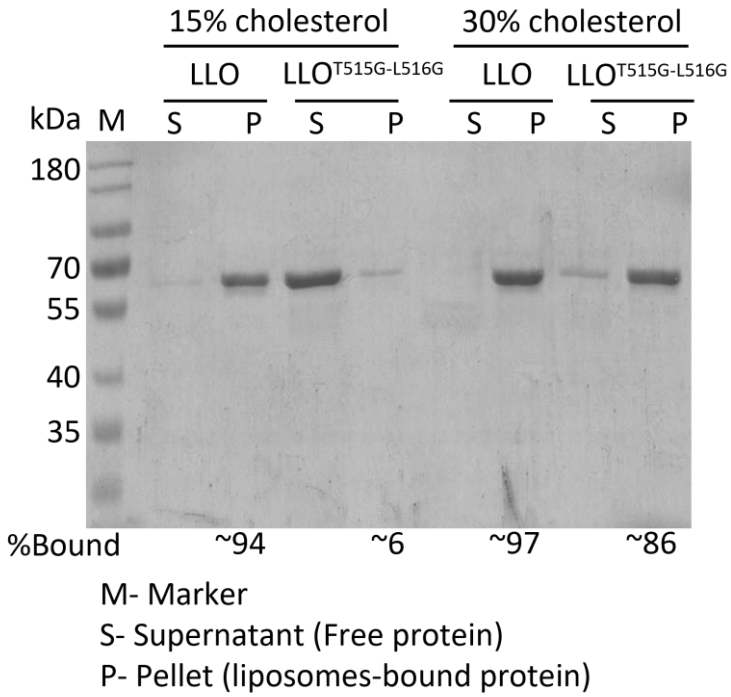

Fig. S7 The image represents the pull down-based binding assay of LLO and LLO$^{T515G-L516G}$, with the PC liposomes containing 15 and 30 wt% cholesterol. Lane M shows molecular weight markers. The percentages of liposome bound LLO and LLO$^{T515G-L516G}$, calculated from the analysis of band intensities using ImageJ software, are indicated below the gel profile.

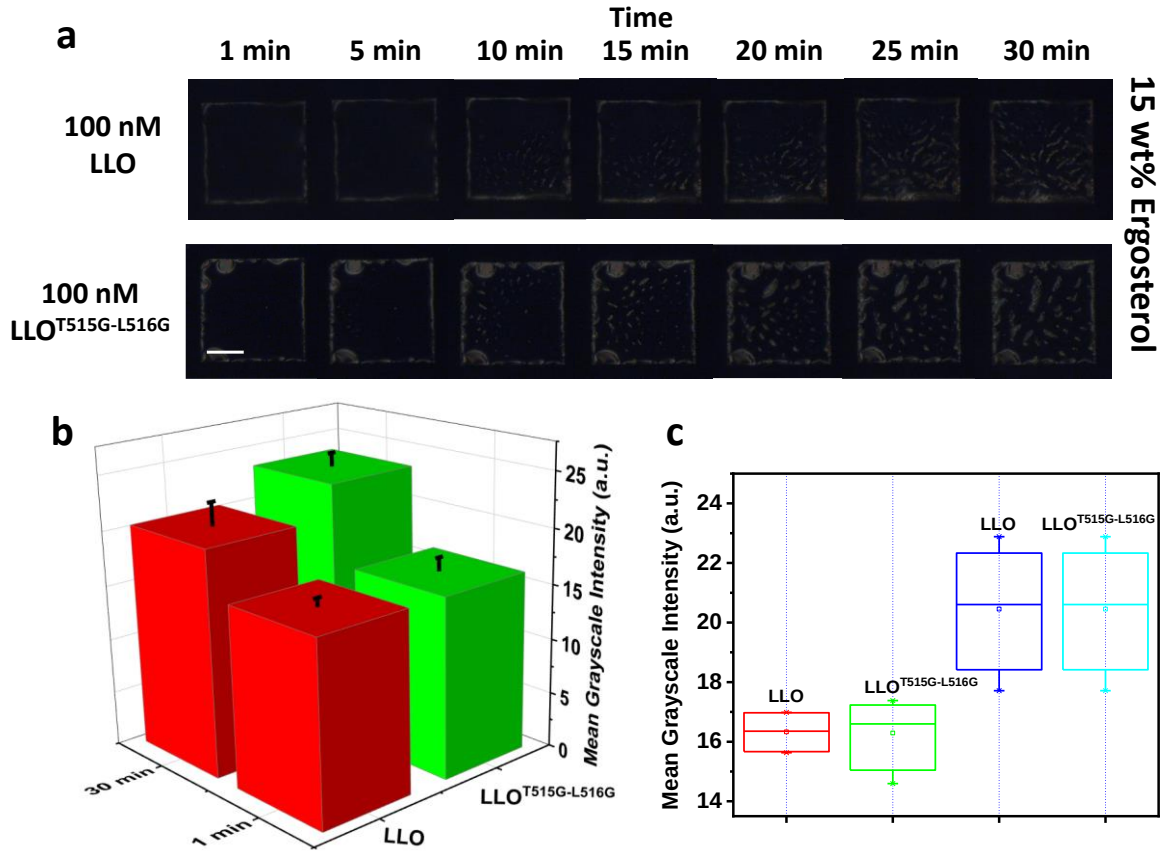

Fig. S8 The optical images in (a) illustrate the time-dependent polarized optical images of LC response in contact with 100 nM proteins at 15 wt% ergosterol/PC lipid-laden LC-aqueous interface. The data demonstrates the negligible difference in LC responses upon incubation of 100 nM LLO and LLO$^{T515G-L516G}$ with 15 wt% ergosterol/PC-laden LC. Scale bar = 100 µm. The bar graph (b) illustrates average mean grayscale intensities (four grid squares) at 1 and 30 min incubation of LLO and LLO$^{T515G-L516G}$ at the designed interfaces. The box plot in (c) illustrates no significant differences (p = 0.9586 > 0.05 (1 min); p = 1 > 0.05 (30 min); Anova) distribution of mean grayscale intensities for LLO and its mutant at 1 and 30 min incubation with PC containing 15 wt% ergosterol, respectively, present at the LC-aqueous interface.

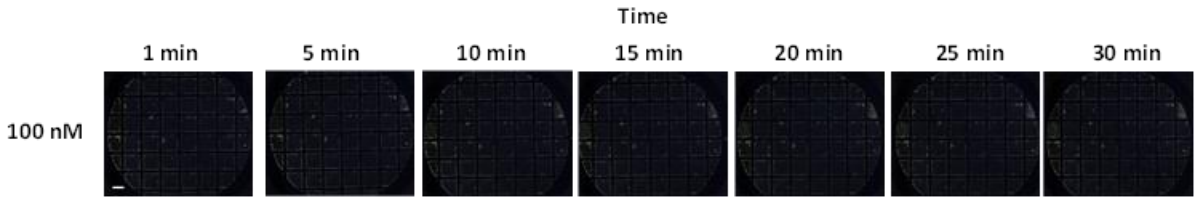

Fig. S9 Time-lapsed polarized optical images of LC response in contact with 100 nM LLO at 0.0425 mg/ml of PC lipid-laden LC-aqueous interface. The data demonstrates the nominal difference in LC responses upon incubation of 100 nM LLO with PC-laden LC. Scale bar = 200 µm.

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