Electronic Supplementary Information (ESI)

Elucidating liquid crystal-aqueous interface for the study of cholesterol-mediated action of a β-barrel pore forming toxin

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EXPERIMENTAL SECTION

MATERIALS

Required Fischer's Finest Premium Grade glass microscopic slides were bought from Fischer Scientific (Pittsburgh, PA). For washing the glass slides, Sulfuric acid, Hydrogen peroxide (30% w/v), Chloroform (HPLC), and Sodium chloride (NaCl) were purchased from Merck. Ethanol was purchased from Jebsen & Jenssen GmbH and Co., Germany (S D fine-chem limited). 4-Cyano-4'-pentylbiphenyl (5CB) liquid crystal, PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 16:0/18:1), Cholesterol, PBS (Phosphate Buffered Saline; pH = 7.2), Tris buffer, thioflavin T (ThT), Dimethyloctadecyl [3-(trimethoxysilyl) propyl] ammonium chloride (DMOAP), Concanavalin A, Fibronectin, Trypsin, 1-(6-(dimethylamino)naphthalen-2-yl)dodecan-1-one, 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan dye), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine-N-(Cyanine 5) [Cy5 labelled VCC], 3β-Hydroxy-5,7,22-ergostatriene (Ergoesterol) were purchased from Merck. Milli-Q water was obtained from Millipore system (Bedford, MA). Coomassie brilliant blue dye and Luria Broth (LB media) were purchased from Himedia. Isopropyl-β-D-Thiogalactopyranoside (IPTG) was purchased from BR Biochem life sciences. Alexa Fluor 488 maleimide was purchased from Thermo Fischer Scientific. The precursor form of *Vibrio cholerae* cytolysin (VCC) i.e., Pro-VCC, was obtained by purification (detailed in methods). Gold Specimen grids (20 µm thick, 50 µm wide, 283 µm grid spacing) were obtained from Electron Microscopy Sciences (Fort Washington, PA).

METHODS

Cleaning of Glass slides

The glass slides were placed in a glass jar, and then freshly prepared piranha solution (70:30 (v/v %) H_2SO_4:H_2O_2) was poured onto them. The whole system was immersed in a water bath whose temperature was maintained at 80 ℃. After 1 h, the glass slides were rinsed with an ample amount of Milli-Q water, dried with a stream of nitrogen gas, and kept in an oven at 100 ℃ for at least 3 h before use.[1] Cleaned glass slides were then immersed in an aqueous solution containing 0.1 % (v/v) DMOAP solutions in DI water for 30 min at room temperature to form a hydrophobic coating over the glass slides. This was followed by washing to remove the excess DMOAP and dried using nitrogen purging. Further, for cross-linking to form a siloxane bond, these slides were put in the oven for 3-6 h.[2]

Preparation of LC-aqueous interfaces

The LC-aqueous interface preparation has already been reported in earlier reports.[1,2] Briefly, DMOAP-coated glass slides were cut into small pieces, and gold grids were placed over them.

Grids were then filled with approximately 0.2 µl of 5CB, and the excess of 5CB was taken out with a syringe to form a uniform film of LC. For the generation of the LC-aqueous interface, the prepared system was placed in an optical well containing (10 mM, 2 ml) PBS buffer.

Preparation of PC vesicles with varying amounts of cholesterol (chol)

PC vesicles or liposomes embedded with different amounts of cholesterol (0%, 10%, 20%, 30%, 40%, and 50% by weight of total lipid) were prepared. For this, the calculated amount of lipid was taken and dissolved in chloroform to make a suspension in a round bottom flask. A required amount of cholesterol (0-50% of cholesterol by weight) was added and then dried under a high vacuum for 3 h at room temperature. After 3 h, PBS buffer was added to the dried lipid, and then freeze-thaw cycles were carried out to prepare large lipid vesicles. Probe sonication of the aqueous lipid dispersion for 30 min resulted in small unilamellar vesicles. All lipid solutions so prepared were used within 24 h.

Purification of VCC

Vibrio cholerae cytolysin (VCC) was recombinantly expressed and purified following the method described in the earlier studies.[3,4] Briefly, the pET14b vector containing the nucleotide sequence corresponding to the precursor form of VCC (pro-VCC) was transformed into the *Escherichia coli* Origami B cells (Merck Millipore). The transformed cells were grown in the LB media at 37 °C till they reached the log phase of growth and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 30 °C. Cells were harvested and recombinantly expressed His-tagged pro-VCC was purified from the soluble fraction of the cell lysate using Nickel-NTA (QIAGEN) affinity chromatography, followed by Q-sepharose (GE Healthcare Life sciences) anion-exchange chromatography. Pro-VCC was converted into the mature form of VCC by proteolytic processing using trypsin (with a protein:trypsin weight ratio of 2000:1). VCC was further purified by another round of anion-exchange chromatography. Homogeneity and purity of the protein were examined by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining. Protein concentration was calculated by measuring absorbance at 280 nm, using the theoretical extinction coefficient determined from the primary protein sequence.

Preparation of lipid-laden LC-aqueous interface

The formed vesicles of lipids were then added to the LC-aqueous interface to form lipid layers over the LC-aqueous interface and incubated for 30 min. After the equilibration period, excess lipid in the solution was removed by washing with PBS buffer (10 mM, pH = 7.2) thrice. Rinsing was performed with high caution so that the lipid assembly and the LC film were not disturbed. Further, to understand protein interactions with the lipid-laden LC-aqueous interface,

the buffer was replaced with varying concentrations of the protein, and the optical response of LC was observed under a polarizing optical microscope (POM). Dispersion of a variable amount of protein was carried out slowly and from the walls of the optical well to ensure no turbulence in the aligned layer of lipid.

Optical Characterization of LC films

The optical characterization has also been reported in prior reports.[2,5] Briefly, the optical response of LC was observed with a POM (Zeiss Scope.A1) in the transmission mode. The optical cell containing the 5CB film was placed on a rotating platform, and each image was focused and captured with a Q-imaging digital camera attached to the POM with an exposure time of 80 ms. Orthoscopic examinations of the orientation of 5CB were done. In all the experiments, images were captured, keeping the source intensity about 40% that of the full brightness.

Emission fluorescence spectra of Laurdan Dye

PC lipid with a variable amount of cholesterol and 0.5 mole % of Laurdan dye were dissolved in HPLC chloroform solution, and then its vesicles were prepared by the aforementioned method. For recording the emission fluorescence spectra, the dye was excited at 360 nm wavelength with an exciting slit width of 1.5 nm, and emission bandwidth was 3 nm. The spectra were recorded within the range of 375 to 650 nm using LabSolutions RF software. Generalized Polarization factor was calculated by using the formula "GP = Generalized Polarization = $(I_{440} - I_{490}) / (I_{440} + I_{490})$", where I_{440} and I_{490} refer to emission intensities at 440 nm and 490 nm, respectively.[6]

Labelling of VCC

VCC was labelled with Alexa Fluor 488-maleimide using the protocol recommended in the manufacturer's kit.[4] Briefly, the VCC protein was first dialyzed in a buffer containing 10 mM Tris, and 400 mM NaCl (pH 7.0). Alexa Fluor 488-maleimide was added drop-wise into the solution containing VCC (keeping the dye:protein molar ratio of 10:1) with constant stirring, and the reaction mixture was incubated at 25 °C for 2 h in the dark. Subsequently, the labelled protein was separated from the free fluorophore using Q-sepharose anion-exchange chromatography. Protein concentration was determined using the Bradford assay.

Confocal Microscope Imaging

To visualize the localization of fluorescently labelled lipid and protein, we have done confocal microscope imaging. Samples were prepared by incubating 20 nM 0.1% Alexa-488 fluorescently labelled VCC with a 2.5% Cy5 fluorescently labelled PC lipid mixed with 0 and 50% of cholesterol. For imaging of sample, a 63 long-distance water immersed lens having

hybrid detectors was used. The scanning speed was 400 Hz, and the image acquisition was set to 1,024×1,024 pixels. Cy5 and Alexa-488 fluorescent dyes were excited with 10% of argon laser power, and laser gain was maintained constant at 5%. A 488 nm laser line was employed for excitation of Alexa-488 labelled VCC, whereas a laser of 650 nm was used for Cy5 excitation. SP8 upright confocal microscope was used to acquire confocal image stacks.

Atomic Force Microscopy (AFM) imaging

For AFM imaging, 20 nM VCC was incubated with the LC film decorated with lipid (PC and 50% cholesterol) for 3 h. After the incubation, the glass slide was taken out, and about 100 µl of PBS buffer (10 mM, pH = 7.2) was poured over the grid to collect the sample in contact with the LC film. Approximately 30 µl of the sample was placed over a cleaned Silicon (Si) wafer and air-dried. After drying, the sample deposited on the Si wafer was flushed with 100 µl of milli-Q water and dried by nitrogen purging for 15 min. The AFM images were taken under tapping mode by Innova Bruker AFM using an antimony-coated silicon tip of 8 nm radius, and the images were processed using WSxM software.[7]

Epifluorescence Imaging of Aqueous-5CB interfaces

Fluorescence imaging was performed using Zeiss (Scope A1) fluorescence microscope as documented earlier.[5] For ThT experiments, the LC-aqueous interface was incubated with a lipid having 50% cholesterol for 30 min. Excess lipid was flushed out, followed by the addition of 20 nM VCC and incubated for 3 h. 5 µM of ThT was added in the end and incubated up to 20 min before imaging.

Quantitative response

The LC optical response at different time points and concentrations were quantified in terms of the gray scale intensity of the optical micrographs. The average gray scale intensity was measured using ImageJ software. The gray scale intensity was averaged for four grid squares of each micrograph to calculate the mean grayscale intensity. A 0.5-15% variation was observed in the gray scale over different sets of experiments.

Supporting Figures

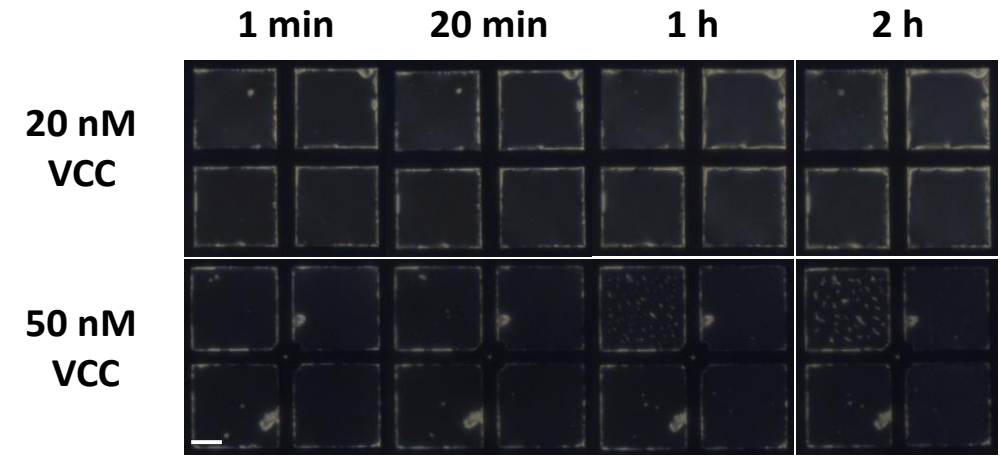

Fig. S1 Optical images of PC-laden LC-aqueous interface upon addition of 20 and 50 nM VCC at different time points up to 2 h. The scale bar is 100 μm.

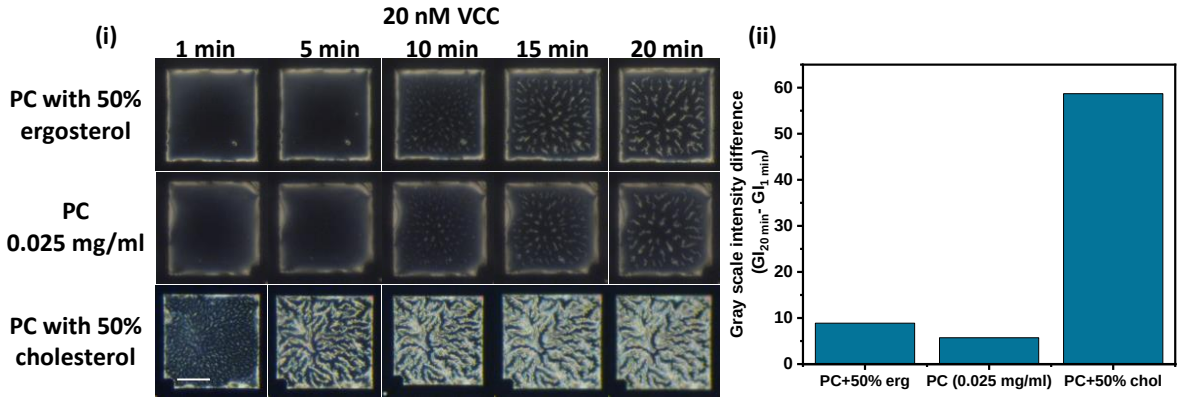

Fig. S2 (i) Dynamic optical images of LC-aqueous interface laden with PC having 50% ergosterol (erg), PC (0.025 mg/ml) and PC having 50% cholesterol upon addition of 20 nM VCC. In all three experiments, the concentration of PC was 0.025 mg/ml. (ii) A plot showing the difference between final (measured at 20 min) and initial gray scale intensities (GI measured at 1 min) i.e., $GI_{20\ min}$- $GI_{1\ min}$ of different lipid-laden LC-aqueous interface in the presence of 20 nM VCC. The scale bar is 100 μm.

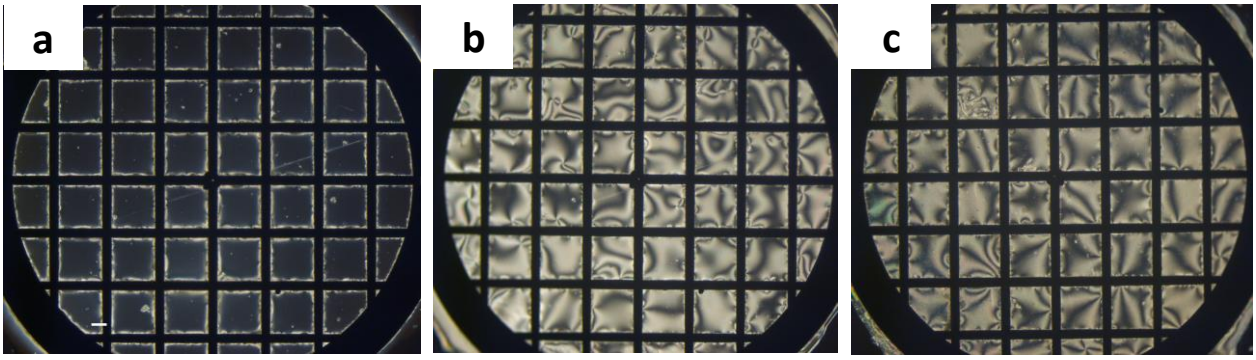

Fig. S3 Optical appearance of (a) LC film encapsulated in gold grid supported over DMOAP-coated glass slide at air interface; (b) LC-aqueous interface; (c) after 2 h incubation of 1 μM VCC at LC-aqueous interface in the absence of lipid. The scale bar is 100 μm.

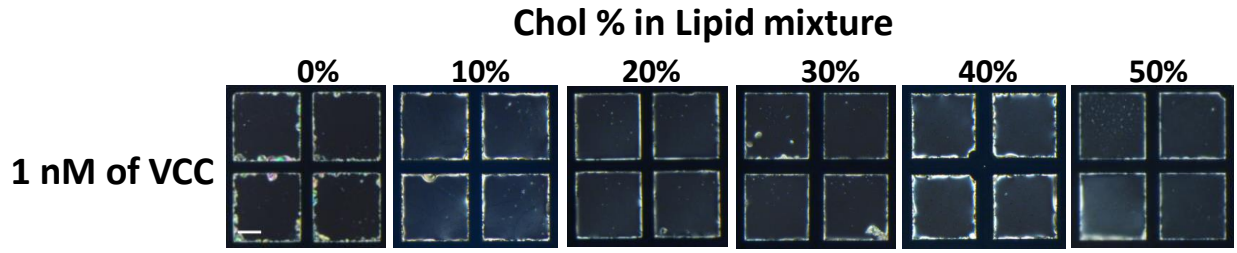

Fig. S4 Optical appearance after incubating 1 nM of VCC with 0.05 mg/ml of lipid having 0-50% of cholesterol for 20 min depicting no change in the LC ordering. The scale bar is 100 μm.

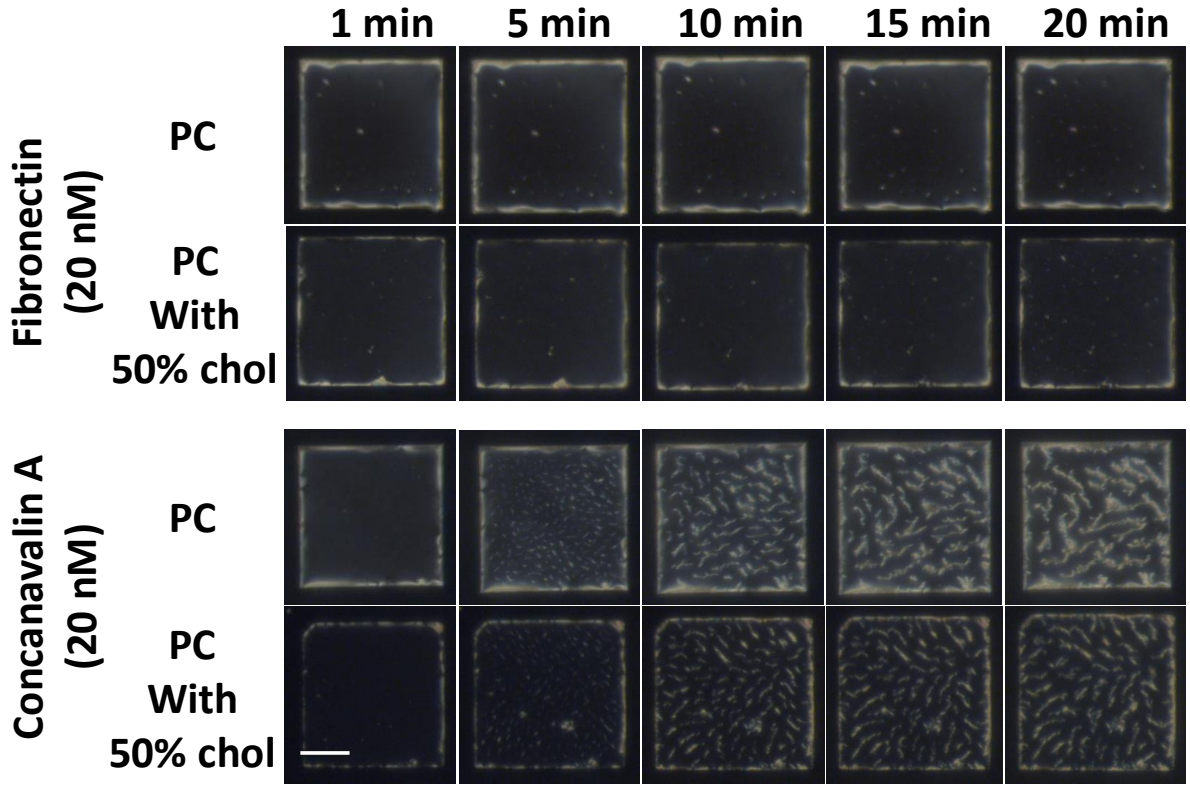

Fig. S5 Optical images of PC and PC/cholesterol mixture laden LC-aqueous interface upon addition of 20 nM of fibronectin and Concanavalin A. Scale bar = 100 μm.

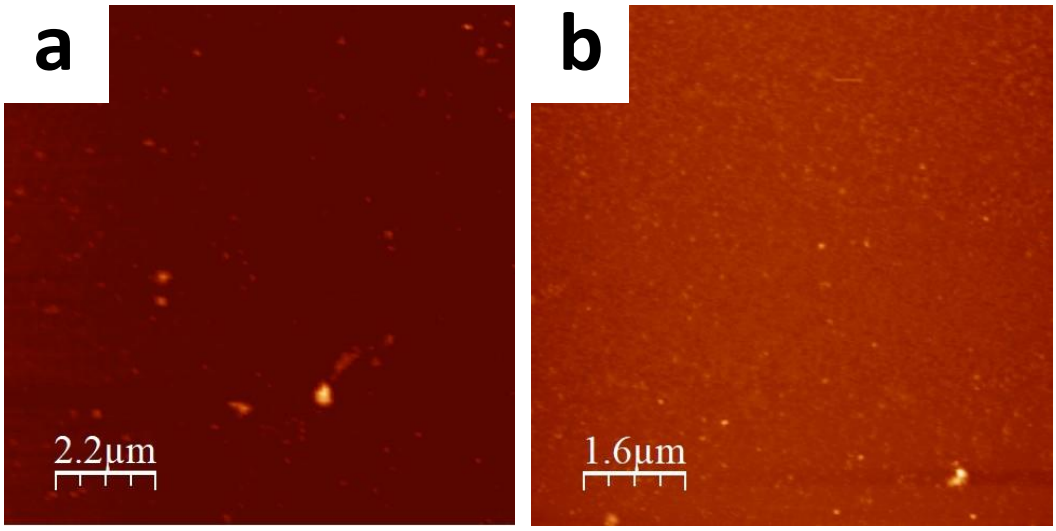

Fig. S6 AFM imaging of samples collected after 3 h (a) from LC-aqueous interface having 20 nM of VCC without lipid; (b) from lipid-laden (PC with 50% cholesterol) without VCC at LC-aqueous interface.

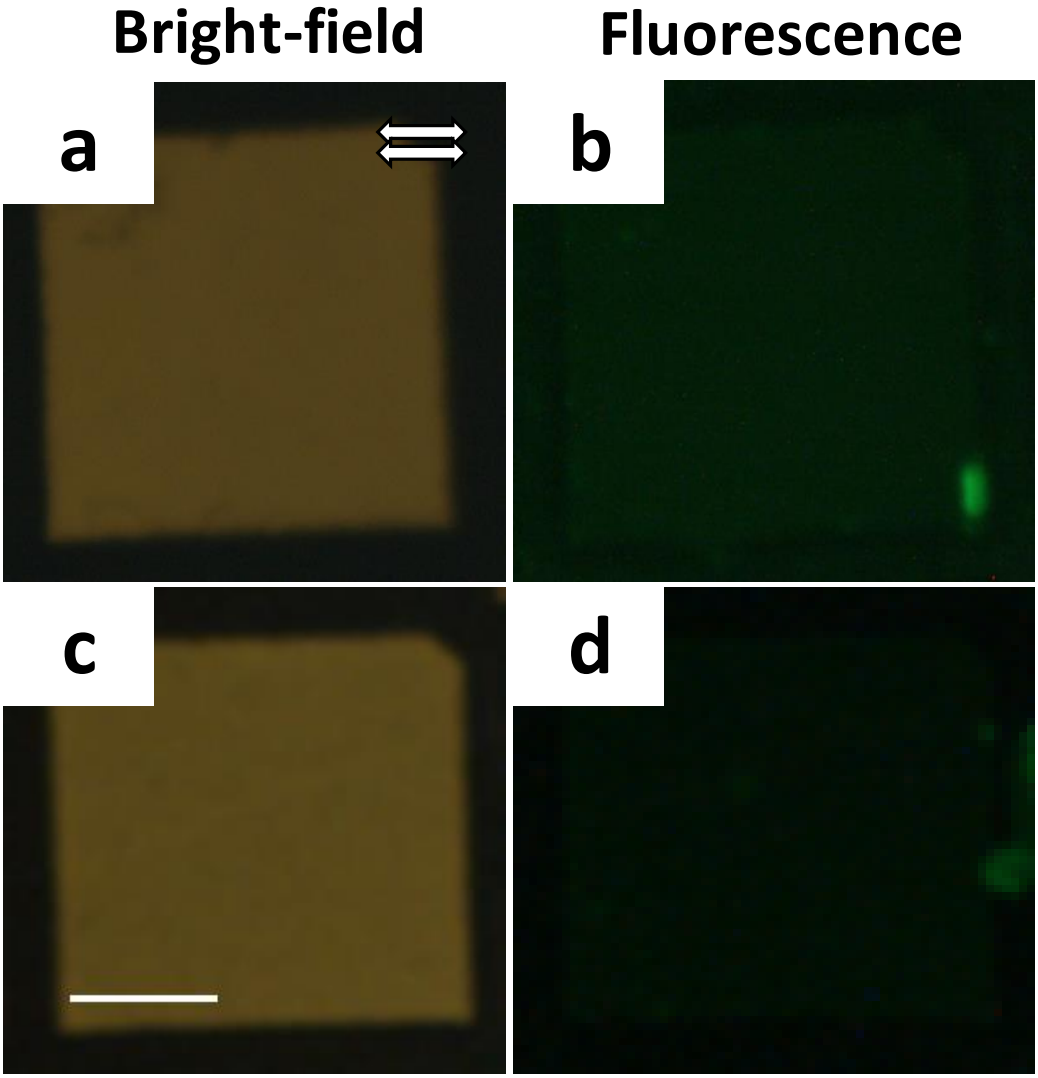

Fig. S7 (a, c) Bright-field, (b, d) respective fluorescence images captured after 3 h incubation of 20 nM VCC without lipid and lipid mixture (PC with 50% cholesterol) without VCC at LC-aqueous interface in the presence of 5 µM ThT, added before the observation. The double-headed arrows on the top right of the image 'a' depict the orientation of polarizers. The scale bar is 100 µm.

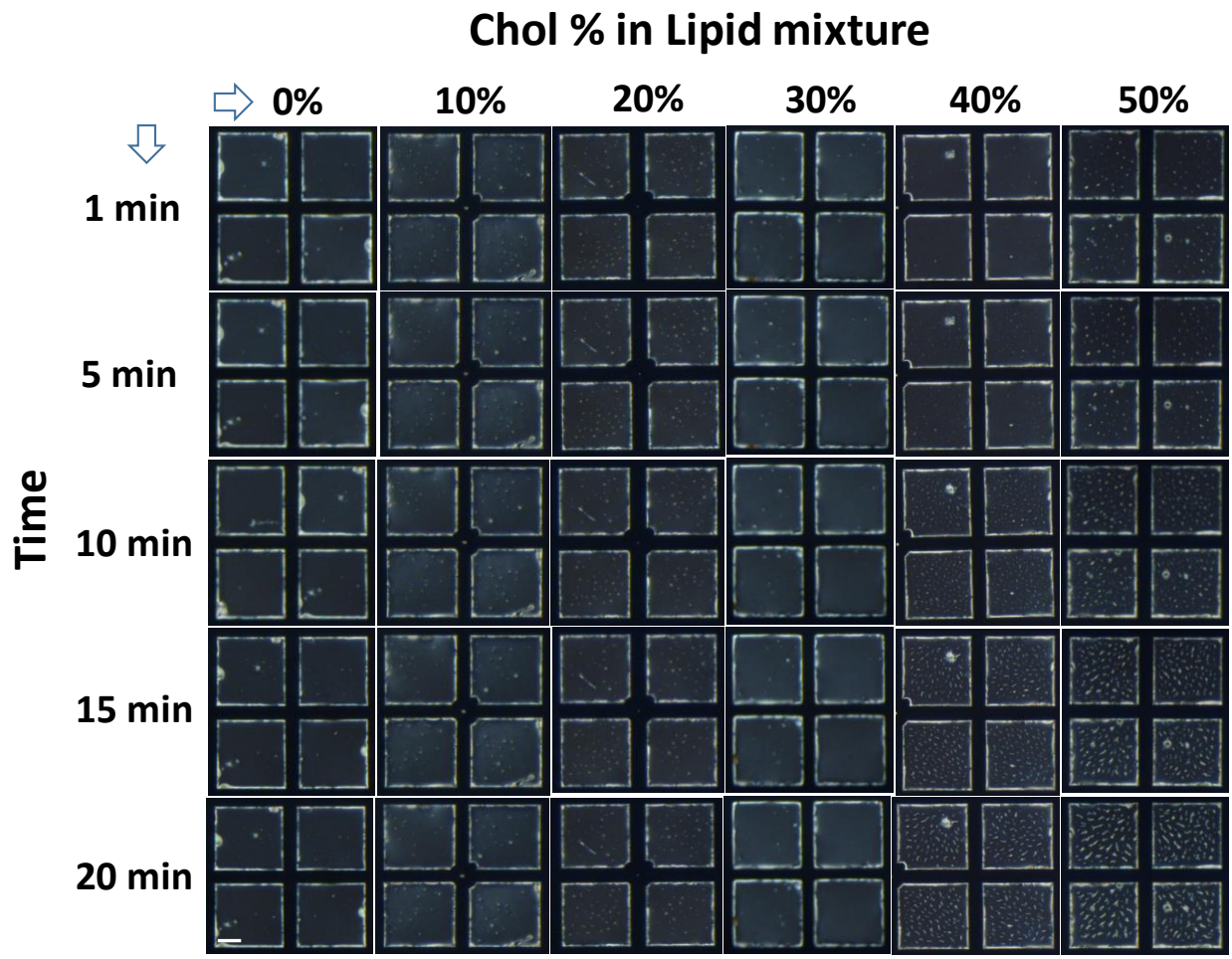

Fig. S8 Dynamic LC response after addition of 3 nM VCC with 0.05 mg/ml of PC embedded with 0-50% cholesterol. The formation of bright domains was observed only in the case of 40 and 50% of cholesterol. The optical micrographs at 20 min are also shown in Fig. 2 in the main manuscript. The scale bar is 100 μm.

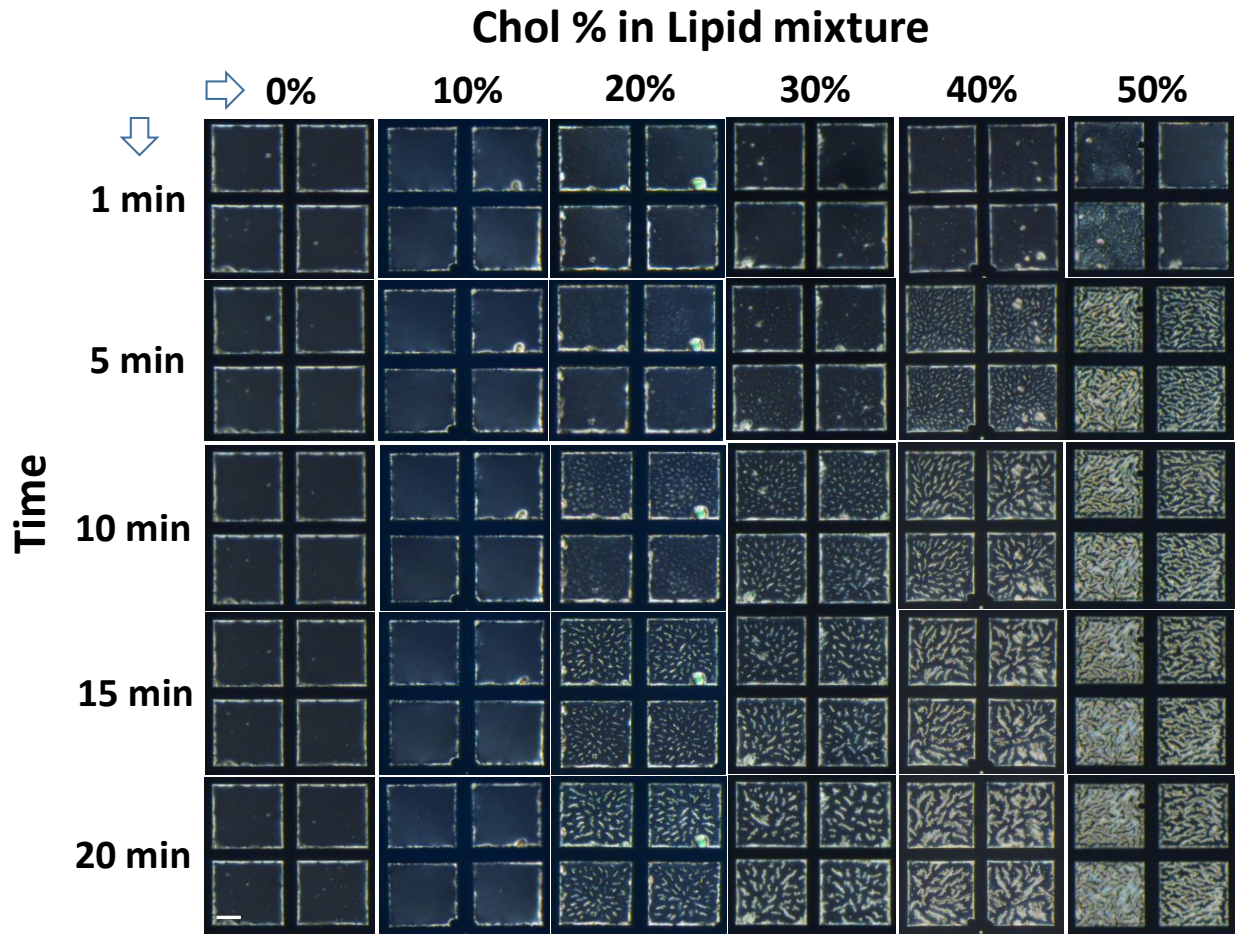

Fig. S9 Dynamic LC response after addition of 10 nM VCC with 0.05 mg/ml of PC embedded with 0-50% cholesterol. The formation of bright domains was observed only in the case of 20-50% cholesterol. The optical micrographs at 20 min are also shown in Fig. 2 in the main manuscript. The scale bar is 100 µm.

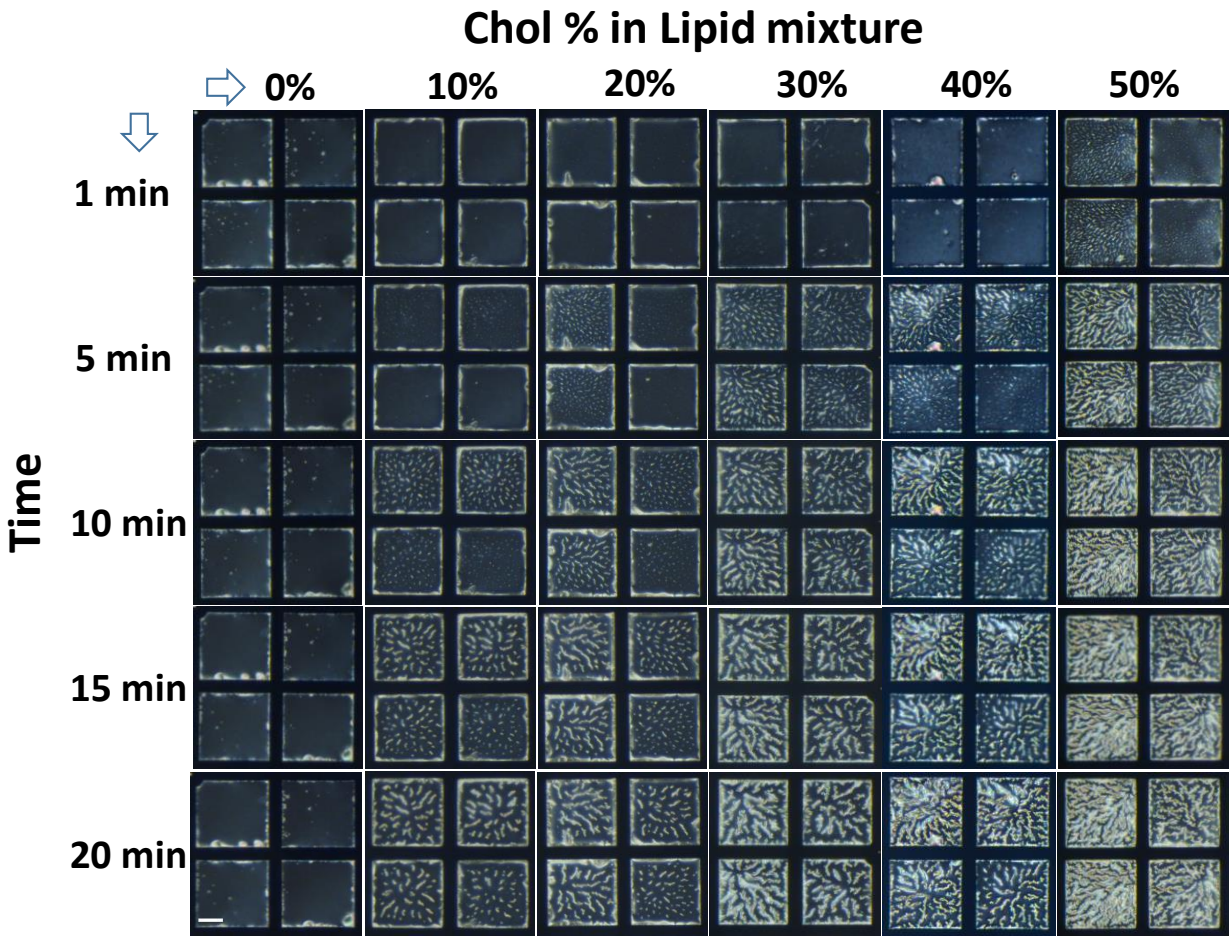

Fig. S10 Dynamic LC response after addition of 20 nM VCC with 0.05 mg/ml of PC embedded with 0-50% cholesterol. The formation of bright domains was observed only in the case of 10-50% cholesterol. The optical micrographs at 20 min are also shown in Fig. 2 in the main manuscript. The scale bar is 100 μm.

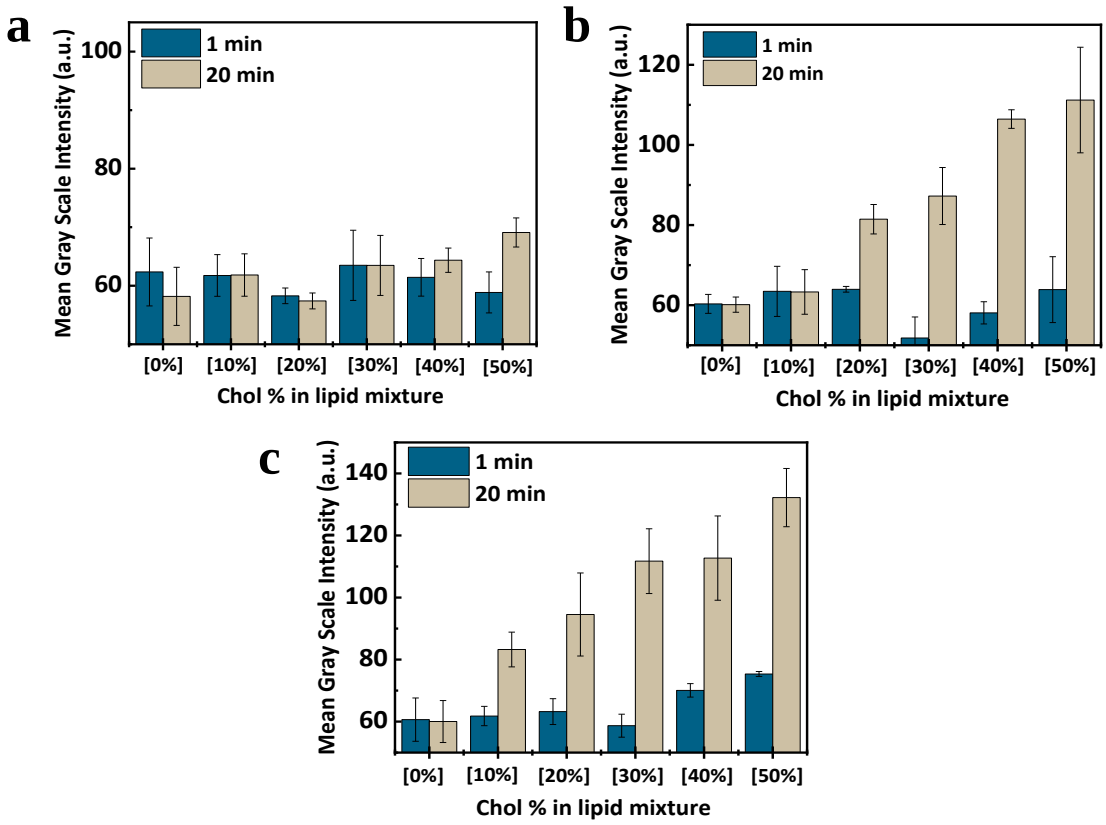

Fig. S11 Comparison of mean gray scale intensities at 1 min and 20 min when PC lipid having variable amounts of cholesterol is exposed to (a) 3 nM, (b) 10 nM, (c) 20 nM amount of VCC protein. The mean gray scale intensities measurements shown in a, b, and c were performed for the optical micrographs shown in Figures S8, S9, and S10. The value indicated in the graph is the average of the mean gray scale intensity of four grid squares.

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