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Ordering Transitions Triggered by Specific Binding of Vesicles to Protein-Decorated Interfaces of Thermotropic Liquid Crystals

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Abstract

We report that specific binding of ligand-functionalized (biotinylated) phospholipid vesicles (diameter = 120 ± 19 nm) to a monolayer of proteins (streptavidin or anti-biotin antibody) adsorbed at an interface between an aqueous phase and an immiscible film of a thermotropic liquid crystal (LC) (nematic 4'-pentyl-4-cyanobiphenyl (5CB)) triggers a continuous orientational ordering transition (continuous change in the tilt) in the LC. Results presented in this paper indicate that, following the capture of the vesicles at the LC interface via the specific binding interaction, phospholipids are transferred from the vesicles onto the LC interface to form a monolayer, reorganizing and partially displacing proteins from the LC interface. The dynamics of this process are accelerated substantially by the specific binding event relative to a protein-decorated interface of a LC that does not bind the ligands presented by vesicles. The observation of the *continuous* change in the ordering of the LC, when combined with other results presented in this paper, is significant as it is consistent with the presence of sub-optical domains of proteins and phospholipids on the LC interface. An additional significant hypothesis that emerges from the work reported in this paper is that the ordering transition of the LC is strongly influenced by the bound state of the protein adsorbed on the LC interface, as evidenced by the influence on the LC of (i) "crowding" of the protein within a monolayer formed at the LC interface and (ii) aging of the proteins on the LC interface. Overall, these results demonstrate that ordering transitions in LCs can be used to provide fundamental insights into the competitive adsorption of proteins and lipids at oil-water interfaces, and that LC ordering transitions have the potential to be useful for reporting specific binding events involving vesicles and proteins.

Introduction

Past studies have established that ordering transitions in thermotropic liquid crystals (LCs) can be triggered by the adsorption and organization of amphiphiles and polymers at interfaces between nematic LCs and immiscible aqueous phases.^{1–5} The surface energetics that control these ordering transitions are remarkably delicate, typically on the order of 1–10 $\mu\text{J}/\text{m}^2$,⁶ thus leading to LC interfacial phenomena that are dependent on the details of the organization of the adsorbates. In addition, because molecules within LC phases are correlated in their orientations over distances of micrometers,⁷ surface-induced ordering transitions in LCs can propagate into the bulk of the LC phases, enabling the reporting of interfacial events through measurements of changes in bulk LC properties (e.g., optical retardance).⁸

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Supporting Informations Available: Experimental details related to the fluorimetric measurements, epifluorescence imaging and optical micrographs of LCs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The assembly of synthetic surfactants and biological lipids at aqueous-LC interfaces has received particular attention in recent studies.¹ For this class of adsorbates, the steric interactions of the tails of the amphiphiles and the mesogens of the thermotropic LC have been shown to couple the interfacial organization of the amphiphiles to the orientational ordering of the LC.^{9–14} For example, contact of an aqueous dispersion of vesicles of dilauroylphosphatidylcholine (DLPC) with the interface of a micrometer-thick film of nematic 4'-pentyl-4-cyanobiphenyl (5CB) has been observed to result in spontaneous formation (via fusion) of a monolayer of DLPC on the interface of the LC, resulting in a discontinuous orientational ordering transition in which the LC changes from an orientation that is parallel to the interface (prior to lipid adsorption) to perpendicular to the interface (after lipid adsorption).⁹ In addition, it was observed that, at interfacial densities of DLPC below saturation coverage, the DLPC monolayer exhibited coexisting lipid-rich and lipid-lean domains which gave rise to patterned orientations of the LC.^{9, 15} A series of subsequent studies established that the phase separation of the DLPC at the interface of the LC was driven by the release of elastic energy stored in the initially strained state of the micrometer-thick film of LC, indicating that LCs should not, in general, be viewed as passive reporters of interfacial phenomena but that they can also be used to direct molecular assembly processes at their interfaces.^{15–17}

All of the studies described above revolve around the adsorption of lipids at unmodified interfaces between aqueous phases and LCs,^{9–11, 15} in which case the adsorption of the lipids is driven largely by hydrophobic interactions with the LC. In contrast, in this paper, we move to examine the interactions of phospholipids with *protein-decorated* interfaces of the LC. Specifically, we sought to determine if specific binding of ligand-functionalized phospholipid vesicles to proteins pre-adsorbed at the aqueous-LC interface would facilitate transfer of phospholipids onto the interface of the LC and thus trigger an ordering transition in the LC. The majority of the experiments reported in this paper were performed with a model system comprised of the protein streptavidin adsorbed onto the aqueous interface of nematic 5CB, and vesicles composed of mixtures of DLPC and biotin-DOPE. In addition, however, we also present experimental results that demonstrate that the principles established using this model system do extend to antibody (IgG)-decorated interfaces of the LC.

Whereas specific binding of phospholipid vesicles to proteins attached at the surfaces of solids has been the subject of a number of past studies,^{18–22} the experimental system reported in this paper differs from those past studies in several key ways. First, in our experimental system, the LC interface onto which the proteins are adsorbed is mobile and deformable.^{10, 23} Thus, upon capture of the vesicles, the proteins and phospholipids at the LC interface are able to reorganize laterally in ways that are not possible at the surfaces of solids. Second, and importantly, the ordering of the LC in our experiments responds to the composition and organization of the proteins and phospholipids captured at the LC interface. In this context, we comment that the interface of a nematic LC is a particularly interesting one because past studies have demonstrated that the influence of adsorbed proteins or lipids on the ordering of the LC is antagonistic.^{18, 24} That is, whereas the interaction of the tails of many lipids (lipids with unbranched tails^{12, 13}) with LCs tends to promote homeotropic (perpendicular) ordering of the LCs (as described above),^{9, 10} past studies have also established that proteins adsorbed to the interfaces of LCs promote planar anchoring of the LCs.^{25–27} In the experiments reported in this paper, we investigated how the competitive interactions of proteins and phospholipids at LC interfaces influence the ordering of LCs.

A central result of the study reported in this paper is that we observe nematic phases of 5CB to undergo *continuous* ordering transitions (continuous change in the tilt of the LC) upon specific binding of vesicles of phospholipid to protein-decorated interfaces of LC. Our

results indicate that, following capture of vesicles at the LC interface via specific binding interactions, phospholipids are transferred from the vesicles onto the LC interface, reorganizing and partially displacing proteins from the LC interface. As discussed later in this paper, the observation of the continuous change in the ordering of the LC is consistent with the presence of small domains of proteins and phospholipids on the interface.²⁸ An additional significant hypothesis that emerges from the work reported in this paper is that the ordering transition of the LC appears to be influenced by the state of the protein adsorbed on the LC interface, as evidenced by the influence on the LC of (i) “crowding” of the protein at the LC interface, and (ii) aging of the protein at the LC interface. Overall, these results demonstrate that ordering transitions in LCs can be used to provide fundamental insights into the competitive adsorption of proteins and lipids at oil-water interfaces, and that the LC ordering transitions have the potential to be useful for reporting specific binding events involving vesicles and proteins. In this context, we note that native vesicles shed by mammalian cells are a largely unexplored source of biological information regarding the states of cells, and that a need exists for analytic methods capable of compositional analysis of native microvesicles.^{29, 30} The interfacial phenomena reported in this paper hints at principles that may form the basis for such methods using LCs.

Experimental Section

Materials

Tris-buffered saline (TBS) (0.05 M TRIS; 0.138 M NaCl; 0.0027 M KCl; pH 8.0), phosphate-buffered saline (PBS) (0.01 M phosphate; 0.138 M NaCl; 0.0027 M KCl; pH 7.4), octyltrichlorosilane (OTS), anti-biotin antibody (produced in goat) and anti-goat IgG antibody were obtained from Sigma-Aldrich (St Louis, MO). Streptavidin, Texas Red-conjugated streptavidin, and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (BODIPY-DHPE) were obtained from Molecular Probes (Eugene, OR). 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (biotin-DOPE) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). 2-propanol and Fisher's Finest Premium Grade glass slides were purchased from Fisher Scientific (Pittsburgh, PA). Gold specimen grids (20 μ m thickness, 283 μ m grid spacing, and 50 μ m bar width) were obtained from Electron Microscopy Sciences (Fort Washington, PA). The nematic LC 4'-pentyl-4-cyanobiphenyl was obtained from EMD Chemicals (Spring Valley, NY). Deionization of a distilled water source was performed with a Milli-Q system (Millipore, Bedford, MA) to give water with a resistivity of 18.2 M Ω -cm.

Methods

Preparation of Protein-Decorated Interfaces of Nematic 5CB—A detailed description of the method used to prepare micrometer-thick films of LC hosted within gold specimen grids can be found in a previous publication.²³ Briefly, glass microscope slides were cleaned according to published procedures and coated with OTS.¹⁸ Gold specimen grids were placed onto the surface of the OTS-treated glass slides. The grids were filled with 5CB using a blunt-tipped glass syringe and the excess LC was removed such that the grid was uniformly filled with LC. The quality of the OTS layer was assessed by checking the alignment of a film of 5CB confined between OTS-treated glass and air. The LC-impregnated grid supported on an OTS-treated glass slide was immersed in a dish of deionized water. Formation of an adsorbed layer of protein on the interface of the LC was accomplished by introducing a solution of streptavidin (1 mg/mL in PBS) into the deionized water in contact with the interface of 5CB. The interface of the LC was incubated against the protein solution for 90 min. At the end of the equilibration period, free streptavidin in the

bulk aqueous solution was removed by sequential exchange or dilution of the aqueous phase with deionized water.

Preparation of Phospholipid Vesicles—Dispersions of vesicles of phospholipid were prepared using previously described methods.¹⁸ Briefly, DLPC and biotin-DOPE (dissolved in chloroform) were dispensed into glass vials. BODIPY-DHPE (dissolved in ethanol) was added when fluorescent measurements were to be performed. The solvents were subsequently evaporated under a stream of N₂ and the vial containing the phospholipids was placed under vacuum for at least 1 h. The dried lipid was resuspended in an aqueous solution of TBS and then extruded several times through a polycarbonate membrane filter (pore size of 100 nm) (Millipore, Bedford, MA). The above-described procedure yielded unilamellar vesicles with an average diameter of 120 nm, as determined using dynamic light scattering (DLS). All phospholipid dispersions were used within 24 h of their preparation.

Interactions of Phospholipid Vesicles with Protein-Decorated Interfaces of

5CB—After formation of protein-decorated aqueous-LC interfaces (as described above), an aliquot of a dispersion of phospholipid was added to the aqueous phase. The LC-filled grids were incubated against the dispersion of vesicles and the orientation of the nematic film of 5CB was then optically characterized according to the methods described below.

Optical Characterization of LC Ordering—The orientation of the nematic phase of 5CB was determined by using plane-polarized light in transmission mode on an Olympus BX60 microscope with crossed polarizers. The gold grid hosting the film of 5CB was placed on a rotating stage located between polarizers. In-plane birefringence was indicated by a bright, colored appearance of the 5CB and the presence of brush textures when the sample was viewed between crossed polarizers.³¹ Homeotropic alignment of the LC was determined by first observing the absence of transmitted light during a 360° rotation of the sample between crossed polarizers. Insertion of a condenser below the stage and a Bertrand lens above the stage allowed conoscopic examination of the LC film. An interference pattern consisting of two crossed isogyres confirmed homeotropic alignment.³² All images were captured using a digital camera (Olympus C-2040 Zoom) mounted on the microscope. The camera was set to an f-stop of 2.8 and a shutter speed of 1/60 s.

Determination of the Tilt of 5CB at the Aqueous-LC Interface—The color of the LC under white-light illumination was matched against a Michel-Levy chart to determine the effective birefringence Δn_{eff} of the 20- μm -thick film of 5CB. For each value of Δn_{eff} , the tilt angle of 5CB at the aqueous-LC interface (measured relative to the surface normal), θ , was determined by solution of the equation

$$\Delta n_{\text{eff}} \approx \frac{1}{d} \int_0^d \left(\frac{n_{\parallel} n_{\perp}}{\sqrt{n_{\perp}^2 \sin^2(\frac{z}{d} \theta) + n_{\parallel}^2 \cos^2(\frac{z}{d} \theta)}} - n_{\perp} \right) dz$$

where $n_{\parallel}$ and $n_{\perp}$ are the indices of refraction parallel and perpendicular to the optical axis of 5CB, respectively, and d is the thickness of the LC film.³³ The indices of refraction of 5CB were taken as constant using the values reported for $\lambda = 632 \text{ nm}$ at 25°C ($n_o = 1.711$ and $n_e = 1.5296$).³⁴ The tilt angles reported in this paper are the average of nine different random locations of each of two independent grids.

Epifluorescence Imaging of Aqueous-5CB Interface—Prior to examination, the aqueous phase in contact with the interface of the LC was diluted with deionized water to

remove free proteins or lipids from the bulk solution. Proteins and phospholipids adsorbed at the aqueous-LC interface were imaged by epifluorescence microscopy using an Olympus IX71 inverted microscope equipped with a 100 W mercury lamp. A fluorescence filter cube with an excitation filter of 560 nm and an emission filter of 645 nm was used to image Texas Red fluorescence. BODIPY fluorescence was imaged using a fluorescence filter cube with an excitation filter of 480 nm and an emission filter of 535 nm. Images were collected with a Hamamatsu 1394 ORCA-ER-CCD camera (Bridgewater, NJ) interfaced to a computer using SimplePCI imaging software (Compix, Inc.). Background fluorescence intensity was determined prior to contact of the LC interface with the fluorescently-labeled biomolecules and subtracted from the raw values. All fluorescence intensity measurements were determined using ImageJ (public-domain image processing software by the U.S. National Institutes of Health).

Quantification of the Interfacial Density of Phospholipid via Fluorimetric Measurements

—Fluorimetric measurements were performed using a FluoroMax-3 fluorimeter (Instruments S. A./Jobin Yvon/Spex Horiba Group, Edison, NJ) with an excitation wavelength of 480 nm (0.5 nm excitation slit) and an emission wavelength range of 490–550 nm (5 nm emission slit) for the detection of BODIPY fluorescence. The fluorimeter was connected to a computer and controlled using DATAMAX software (Instruments S. A./Jobin Yvon/Spex Horiba Group).

To determine the interfacial concentration of phospholipid at the aqueous-LC interface, the following procedure was used.³⁵ First, a calibration curve of fluorimetric intensity versus known lipid concentration in bulk solution was prepared by using 1 mL of 2-propanol containing known amount of phospholipids (containing 1 mol % BODIPY-DHPE). Next, phospholipid-decorated 5CB was extracted from a metal grid using a blunt-tip micro-syringe and dissolved in 1 mL of 2-propanol. Fluorimetric measurements were used to determine the amount of phospholipid in each sample. When combined with knowledge of the area of the LC interface, the above measurements were used to calculate the interfacial concentration of phospholipid in each experiment.

Results

Adsorption of Streptavidin at Aqueous-5CB Interfaces

While many past studies have reported that amphiphilic polymers and lipids adsorb spontaneously onto aqueous-LC interfaces^{1, 2} (and that proteins adsorb to isotropic oil-water interfaces^{36–38}), the adsorption of proteins onto aqueous-thermotropic LC interfaces has not been widely characterized.³⁹ Initially, therefore, we performed a series of fluorescence measurements to determine the extent to which streptavidin adsorbs from bulk aqueous solution onto aqueous interfaces of nematic 5CB. We also investigated whether the ordering of the nematic 5CB was measurably perturbed by adsorption of the streptavidin. In these experiments, an aqueous-5CB interface was prepared (see Methods) and incubated against a buffered (PBS) solution of streptavidin comprised of 1 mol % Texas Red-conjugated streptavidin for 90 min. The aqueous solution of streptavidin was subsequently exchanged with deionized water via sequential dilution to remove the free streptavidin from bulk solution. The resulting LC interface was imaged for Texas Red fluorescence. Figures 1a and 1b show fluorescent micrographs of aqueous-5CB interfaces that were incubated against either a solution free of streptavidin (Figure 1a) or a 1 μ M solution of streptavidin (Figure 1b). Inspection of Figures 1a and 1b reveals that the interface that was incubated against the solution containing streptavidin (Figure 1b) exhibited a fluorescence intensity that was higher than the control (Figure 1a), confirming that streptavidin adsorbed onto the LC interface. As noted above, this result is generally consistent with past studies of protein adsorption onto

oil-water interfaces.^{36–38, 40} In addition, we note that the pI of streptavidin is between 5 and 6.⁴¹ At the pH of our experiments (7.4), we expect, therefore, streptavidin to carry a net negative charge. Because the ζ -potential of a 5CB-water interface at pH 7.0 has been measured to be -50 mV,⁴² the adsorption of streptavidin to the interface of 5CB, as reported above, is likely driven by forces other than electrostatic ones (e.g., hydrophobic interactions).

Figures 1c and 1d show polarized light micrographs of the nematic films of 5CB following contact with the aqueous solutions without or with streptavidin, respectively. These optical micrographs (crossed polars) reveal the LC to exhibit a bright and colorful optical appearance under white-light illumination. Here, we briefly sketch the basis of our interpretation of these and other polarized light micrographs presented in this paper (we refer the reader to past publications for detailed discussions^{2, 23}). In the experiments reported in this paper, the LC is supported on and oriented perpendicular to a monolayer of OTS formed on a glass slide.¹⁸ By using the Michel-Levy color chart to quantify the retardance of the $20\text{-}\mu\text{m}$ -thick, supported film of 5CB, the optical appearance (with pale green-pink or yellow hues) of the LC in Figure 1c can be used to establish that the LC is anchored parallel to the aqueous-5CB interface.³² These so-called hybrid anchoring conditions introduce splay and bend strain into the film of LC (see Figure 1e for the director profile). We also note that a prominent feature of the optical appearance of the LC within each compartment of the metallic grid used to stabilize the film of LC is the presence of dark brushes.⁴³ This feature is caused by variation of the azimuthal orientation of the LC within each compartment. A comparison of Figure 1c and 1d reveals that adsorption of streptavidin onto the interface of the LC does not lead to a significant change in the optical appearance of nematic 5CB (colors or defect/brush textures). This result is consistent with previous studies which have concluded that protein-decorated surfaces of *solids* give rise to planar orientations of nematic 5CB.^{25–27}

We also quantified the effect of the concentration of streptavidin in the bulk aqueous solution (1 mol % Texas Red-conjugated streptavidin) on the resulting density of streptavidin adsorbed onto the LC interface. Figure 1f shows that the fluorescence intensity of Texas Red-conjugated streptavidin at the LC interface increases with increasing concentration of streptavidin within the bulk solution contacted with the LC interface (for 90 min). In addition, incubation of the LC interface against $1\text{ }\mu\text{M}$ of streptavidin for 24 h did not result in an increase in epifluorescence intensity relative to the highest value ($10\text{ }\mu\text{M}$) reported in Figure 1f, suggesting that a fluorescence intensity of 120 A.U. in Figure 1f does correspond to saturation (monolayer) coverage. Below, we further demonstrate that the adsorption of streptavidin at these LC interfaces is irreversible (unless incubated against phospholipid).

Orientational Transitions in 5CB Induced by Specific Capture of Phospholipid Vesicles

Following verification that the LC interfaces used in our study were decorated with streptavidin (see above), we sought to determine if capture of biotinylated vesicles via specific binding to the streptavidin-decorated interface of the LC would induce an ordering transition in the LC that is distinct from that seen when the binding groups were absent from either the vesicles or the LC interface.

In the absence of proteins bound at the LC interface (i.e., unmodified LC interface), incubation of the LC interface against a dispersion of vesicles of DLPC (no biotin-DOPE) resulted in the nucleation and growth of dark domains (when observed between crossed polars) (Figure 2a, 10 min). The dark domains correspond to regions of homeotropic anchoring of the LC, a result of fusion of vesicles with the interface of the LC and transfer of phospholipid onto the LC interface (Figure 3a).⁹ As noted in the Introduction, Gupta *et al.*

subsequently showed that the elasticity of the nematic LC drives the lateral organization of the lipid on the interface into micrometer-sized domains in a manner that minimizes the elastic energy stored in the film of LC.¹⁵ In our experiment, a uniform dark appearance of the LC (absence of in-plane birefringence) was achieved after incubation of the LC interface against the dispersion of DLPC vesicles for 20 min (Figure 2a). We note that each of the dark squares in the grid in Figure 2a is framed by a bright edge. The bright edge is the result of perpendicular anchoring of the LC on the vertical walls of the grid.

Next, we determined if the presence of streptavidin adsorbed onto the LC interface (from a 1 μ M aqueous solution of streptavidin) would alter the dynamic ordering transition of 5CB upon exposure to a dispersion of vesicles in the absence of the biotin-DOPE binding groups within the vesicles. Incubation of the streptavidin-decorated interface against a dispersion of vesicles comprised of DLPC resulted in a slow but continuous progression of interference colors (Figure 2b), a consequence of a continuous change in the tilt of the LC at the aqueous-5CB interface over > 90 min. Because the LC assumes a planar orientation at a protein-decorated interface (Figure 1d), whereas phospholipids at the interface induce a homeotropic orientation (Figure 2a), we interpret the tilting of the LC to reflect the competitive influence of proteins and phospholipids at the interface on the LC (Figure 3b). Here we also note that a similar, continuous change in the tilt of the LC was observed when a dispersion of biotinylated vesicles (5 mol % biotin-DOPE) was incubated against an aqueous-5CB interface that was decorated with a protein that does not bind biotin (non-specific IgG) (Figure 2c).

Next, we investigated if *specific binding* of biotinylated vesicles to the streptavidin-decorated interface of a LC would trigger an ordering transition that was distinct from the above-described cases that did not involve specific binding events. To this end, a dispersion of biotinylated vesicles containing 5 mol % biotin-DOPE was incubated against a streptavidin-decorated interface of 5CB. In the presence of the specific binding interaction, we observed a continuous transition in the orientation of the LC (Figure 2d) with dynamics that were accelerated substantially compared to that observed in the absence of specific binding interactions (Figure 2b and 2c). Specifically, whereas the LC did not reach a homeotropic orientation for the latter cases for at least 90 min, homeotropic anchoring was evident within 30–40 min of incubation of biotinylated vesicles against the streptavidin-decorated aqueous-LC interface.

To quantify the difference in the orientations of the LC induced by specific and non-specific interactions of the vesicles, the interference colors generated by the LC under white-light illumination were used to determine the tilt of the LC at the aqueous interface as a function of time (Figure 2e). These results clearly reveal that specific binding of vesicles to the protein-decorated LC interface leads to an accelerated ordering transition in the LC. Indeed, inspection of Figure 2e reveals that the ordering transition induced by the specific binding event is almost complete prior to the onset of the ordering transition induced by non-specific interactions. Overall, we interpret these results to suggest that the biotin-mediated capture of vesicles at the LC interface serves to concentrate vesicles near the LC interface, and that following localization of the vesicles near the interface (see below for evidence of lipids at the LC interface), there is spontaneous transfer of phospholipids onto the LC interface to trigger the ordering transition. As noted above, this interpretation is supported by past studies that have shown that the presence of phospholipids at the aqueous-LC interface causes homeotropic anchoring of the LC.^{9, 10} As described below, however, an additional necessary process underlying the LC ordering transition is partial displacement of protein from the LC interface (by the lipids).

The above results lead to two important additional conclusions. First, we conclude that the presence of streptavidin adsorbed at the LC interface does not prevent but greatly retards phospholipid adsorption relative to the protein-free interface of the LC (from < 10 min to > 90 min). Such retardation of the LC ordering transition by the presence of interfacial proteins was also seen when an aqueous-LC interface decorated with streptavidin was exposed to sodium dodecyl sulfate (SDS) (Figure S1). Second, in contrast to the protein-free interface of the LC, for which the LC ordering transition was discontinuous and micrometer-sized domains of patterned LC were evident (Figure 2a), the LC ordering transition induced by phospholipid adsorption in the presence of interfacial proteins was continuous. As discussed in more detail below, this continuous tilting of the LC orientation hints that the presence of the protein on the LC interface limits the size of the phospholipid domains to sub-optical sizes. Connected to this proposition, here we note also that the molecular-level mixing of phospholipids and proteins on the LC interface (no domains) would be expected to lead to a discontinuous transition in the orientation of the LC.^{28, 44}

Quantification of Proteins and Phospholipids at the Aqueous-5CB Interface

To provide additional insight into the results described above, we quantified the interfacial density of phospholipid captured at the LC interface, along with the change in interfacial density of streptavidin at the LC interface following incubation against the dispersions of vesicles. Fluorescently-labeled vesicles (1 mol % BODIPY-DHPE) and proteins (1 mol % Texas Red-conjugated streptavidin) were used in these studies. The protein-decorated interface of the LC was imaged prior to and following exposure for 90 min to a dispersion of either biotinylated or non-biotinylated vesicles (after flushing with TBS).

First, we sought to test our hypothesis that the accelerated ordering transition of 5CB triggered by specific binding of biotinylated vesicles to streptavidin adsorbed at the LC interface was the result of a biotin-mediated increase in local concentration of phospholipids near the interface. Inspection of Figure 4a reveals the BODIPY fluorescence at the LC interface that had been in contact with biotinylated vesicles to be substantially increased. By performing quantitative fluorimetric measurements of phospholipids extracted from the LC interface (see Methods), following incubation of the streptavidin-decorated LC interface against biotinylated vesicles, the density of interfacial phospholipid was determined to be 4.7 ± 0.5 molecules/nm² (Figure 4b). We note that this density of lipid is greater than monolayer coverage, a point that we return to below.¹⁰ A far smaller phospholipid density (0.3 ± 0.1 molecules/nm²) was measured for the interface that was incubated against biotin-free vesicles. These results are consistent with a physical picture in which specific binding of the biotinylated vesicles to streptavidin adsorbed at the LC interface leads to a higher local concentration of phospholipids relative to that observed in the absence of the specific binding interaction, which, in turn, is transduced via the ordering transition of the LC.

We also investigated whether association of the phospholipid with the LC interface (and perturbation of the anchoring of 5CB) was accompanied by redistribution or displacement of the streptavidin from the interface. Figure 4c shows that the density of streptavidin (as inferred by the fluorescence intensity of 1 mol % Texas Red-conjugated streptavidin at the interface) was measured to decrease at the LC interface that was incubated against biotin-free vesicles ($25 \pm 7\%$). The decrease was greater, however, when the vesicles were biotinylated ($46 \pm 4\%$). Since the decrease in interfacial density of streptavidin (Figure 4c) coincides with an increase in interfacial concentration of phospholipid (Figure 4a and 4b), we interpret these results, when combined, to suggest that the displacement of streptavidin from the LC interface is driven by phospholipid adsorption. This interpretation is further supported by the observation that the streptavidin-decorated LC interface, when incubated against buffer free of vesicles for 90 min, exhibited negligible loss of Texas Red fluorescence. Additionally, we conducted fluorimetric measurements using the bulk aqueous

solutions against which the streptavidin-decorated interfaces of the LC had been incubated to assay for the presence of any Texas Red-conjugated streptavidin displaced from the interface into the bulk solution (Figure S2). A higher Texas Red fluorescence intensity was measured in the dispersion of biotinylated vesicles as compared to the dispersion biotin-free vesicles (154 ± 45 vs 48 ± 8 A.U.), providing further support for our conclusion that streptavidin was displaced by phospholipid adsorbing to the LC interface. We also note that the displacement of streptavidin from the interface is not complete (i.e., not all the fluorescence signals from the labeled streptavidin was lost). As discussed below, it is likely that some streptavidin is bound to biotinylated lipid on the LC interface (via biotin-streptavidin binding) or co-exists along with phospholipid in an adsorbed state on the LC interface.⁴⁵

Aging of Protein Adsorbed at the LC Interface

As an additional test of the proposition that protein displacement from the LC interface is a necessary step in the above-described ordering transitions triggered by biotinylated vesicles, we investigated the effect of aging of the streptavidin on the LC interface on the LC ordering transition. Past studies have demonstrated that the interfacial shear viscosity of a protein film increases with age due to conformational changes and physical cross-linking of proteins on the interface.^{46, 47} In addition, adsorbed layers of proteins, when aged, have also been documented to produce interfacial elastic films that are displaced only by application of large external surface pressures.⁴⁸ We hypothesized, therefore, that aging of the streptavidin would produce a state of the adsorbed protein that would be less readily displaced by phospholipid, resulting in a slower/retarded ordering transition of the LC. To test this hypothesis, we incubated the aqueous-5CB interface against a $1 \mu\text{M}$ solution of streptavidin for 90 min, rinsed the interface, and then left it overnight to “age” prior to exposure to a dispersion of vesicles (5 mol% biotin-DOPE). A control experiment conducted using Texas Red-conjugated streptavidin (1 mol %) verified that the fluorescence intensity of Texas Red at the LC interface remained constant after the overnight incubation, indicating negligible loss of streptavidin from the LC interface (Figure S3).

A comparison of Figures 5a and 5b reveals that the LC ordering transition triggered by specific binding of biotinylated vesicles was, indeed, retarded by aging of the interfacial streptavidin. Epifluorescence measurements also showed that the decrease in signal from Texas Red-conjugated streptavidin at the aged interface (following incubation against the biotinylated vesicles for 60 min) (15 ± 2 %) was less than that observed at the streptavidin-decorated interface that was not aged (34 ± 2 %) (Figure S3). These results thus provide support for our conclusion that the LC ordering transition induced by specific binding of biotinylated vesicles involves displacement of streptavidin from the LC interface, and that an aged protein layer at the aqueous-5CB interface slows the displacement process when exposed to phospholipid (Figure 3c). As noted above, a number of past studies have concluded that aged protein films become increasingly difficult to displace from interfaces using amphiphiles.^{47, 49}

Effect of Interfacial Density of Streptavidin

Next, we examined how the density of streptavidin adsorbed onto the aqueous-5CB interface influenced the LC ordering transitions induced by binding of biotinylated vesicles. Based on the hypothesis described above, we predicted that an increase in the interfacial density of streptavidin would inhibit incorporation of the phospholipids onto the LC interface, and thus slow the LC ordering transition. Inspection of Figure 6, however, reveals that the LC ordering transition induced by specific binding of the biotinylated vesicles was *accelerated* with increasing interfacial density of streptavidin (Figure 6a–d). This result is, at first sight, seemingly contradictory of the above-described hypothesis. The trend seen in Figure 6a–d

was also seen when the LC interface was exposed to biotin-free vesicles. That is, streptavidin adsorbed to the LC interface from a solution of high streptavidin concentration gave rise to a fast ordering transition when the interface was incubated against non-biotinylated vesicles (Figure S4). We note, however, that past studies have also demonstrated that crowding of proteins within an adsorbed layer (leading to lateral, steric interactions) can impact the conformational state of the protein at the interface. That is, when the interfacial density of protein at an interface is high, space available for the protein to spread and unfold at the surface is low.^{50, 51} As mentioned above, unfolding of proteins permits intermolecular interactions between neighboring proteins, as well as stronger adsorption of proteins to the interface.⁵⁰ Thus, we interpret the results described above to suggest that the ordering transition of 5CB induced by binding of vesicles to the LC interface is accelerated at high interfacial densities of streptavidin due to either weaker inter-protein associations and/or weaker adsorption of the proteins onto the LC interface, both of which permits facile displacement of proteins from the interface by phospholipids (Figure 3b and 3c). This interpretation is consistent also with the above-noted effects of aging of the proteins on the LC interface.

In support of the above-described interpretation, Figure 6f shows the change in fluorescence intensity of Texas Red-conjugated streptavidin at the LC interface following incubation against dispersions of biotinylated vesicles, as a function of the interfacial density of streptavidin. This data reveals that the loss of streptavidin from the LC interface (inferred from the decrease in Texas Red fluorescence intensity) is indeed more pronounced at the higher initial interfacial densities of streptavidin. We also note that the amount of streptavidin adsorbed onto the LC interface from solutions containing intermediate bulk concentrations is low (e.g., for 0.1 μM in Figure 6f, we estimate the coverage of protein to be $\sim 10\%$ of saturation coverage). Interestingly, the presence of this low interfacial concentration of protein is sufficient to retard the ordering transition of the LC upon incubation against dispersions of biotinylated vesicles (Figure 6c). As noted above, we infer that the state of the bound protein (strongly bound and likely partially unfolded) hinders adsorption of phospholipids onto the LC interface at the low coverage evident in Figure 6 (see also Figure 3c).

When the density of streptavidin adsorbed to the LC interface was less than a few percent of saturation coverage (prepared from a solution of 0.001 μM), we observed incubation of the LC interface against biotinylated vesicles to cause the nucleation and growth of homeotropic regions of LC (not a continuous change in the tilt of the LC) (Figure 6e). For these interfaces, by using epifluorescence imaging, we observed the lateral segregation of streptavidin (1 mol % Texas Red-conjugated) (Figure S5). Specifically, we established that the homeotropic domains of LC corresponded to lipid-rich regions (appears bright when imaged for BODIPY-DHPE) and the co-existing planar LC domains corresponded to protein-rich regions of the interface. This result suggests that at sufficiently low concentrations of streptavidin, lateral reorganization of phospholipid and protein on the interface leads to lipid- and protein-rich domains on micrometer and larger scales (Figure S5).

Finally, we quantified the amount of phospholipid captured at the LC interface as a function of interfacial density of streptavidin. Figure 6g indicates that the amount of phospholipid captured at the interface increases with density of interfacial streptavidin, such that the LC interface with the highest density of streptavidin captures roughly twice the amount of phospholipid captured at the LC interface with the lowest density of streptavidin. Since a phospholipid density of 4.7 ± 0.5 molecules/ nm^2 was measured at the LC interface that had been incubated with 1 μM of streptavidin, we conclude that the amount of phospholipid captured in the interfacial region under all conditions exceeds monolayer coverage (~ 2

molecules/nm²) and thus is sufficient, in principle, to trigger homeotropic ordering of the LC if adsorbed onto the LC interface.¹⁰ The slow LC response at intermediate densities of streptavidin (0.1 and 0.01 μ M, Figure 6c and 6d) is, therefore, not due to insufficient phospholipid in the interfacial region. We conclude that it is likely that the phospholipid exists at the LC interface in at least two states.⁴⁵ One state of the phospholipid interacts with the LC, and thus contributes to the observed ordering of the LC. A second population of phospholipid, while bound to the interfacial region, likely does not interact directly with the LCs but is bound to the streptavidin at the LC interface (e.g., adsorbed vesicles). This proposition is supported by our measurement of the presence of lipids (indicated by BODIPY fluorescence) in regions of the LC interface that exhibit planar LC orientations (Figure S5).

Ordering Transitions at Interfaces of 5CB Decorated with Anti-biotin Antibody

We end this manuscript by reporting that the interfacial phenomena described above using biotin and streptavidin are also seen when the LC is decorated with proteins other than streptavidin. Specifically, we prepared an anti-biotin antibody-decorated interface of 5CB by incubating an aqueous-LC interface against a buffered solution containing 0.05 mg/ml of the antibody. Figure 7 shows that, similar to a streptavidin-decorated LC interface, an accelerated ordering transition of LC was observed when the anti-biotin antibody-decorated LC interface was incubated against a dispersion of biotinylated vesicles (relative to a control experiment using biotin-free vesicles). The LC ordering transition also occurred via a continuous change in the tilt of the LC. When the interfacial density of the anti-biotin antibody at the LC interface was decreased (by decreasing the concentration of the antibody within the bulk aqueous solution against which the LC interface was incubated), the ordering transition induced by binding of biotinylated vesicles was retarded (Figure S6), similar to the result shown in Figure 6 with the streptavidin-decorated LC interfaces. Overall, this experiment illustrates that ordering transitions in LCs can be induced by specific binding of vesicles via antibody-antigen interactions. We conclude that the interfacial phenomena reported in this manuscript are potentially generalizable to a range of other receptor-ligand interactions involving vesicles.

Discussion

One of the key findings of the study reported in this paper is that the antagonistic influence of adsorbed proteins and phospholipids on the orientational ordering of LCs can, under some circumstances, give rise to continuous ordering transitions in LCs. As mentioned above, a number of past studies have established that the hydrocarbon tails of phospholipids play a dominant role in dictating the homeotropic orientation of LCs at lipid-laden LC interfaces.^{9, 10} In particular, early studies concluded that lipid tail length and areal density are important determinants of the ordering of LCs by lipids.⁵² Unlike phospholipids, most proteins do not possess long alkyl tails and therefore do not cause homeotropic alignment of LCs. Indeed, while elucidation of the intermolecular interactions responsible for anchoring of LCs on protein and peptide-decorated interfaces is an ongoing area of research,⁵³ most protein-functionalized surfaces give rise to planar anchoring of LCs.^{25–27}

The *continuous* nature of the LC ordering transitions induced by binding of vesicles to protein-decorated LC interfaces, as reported in this paper, is significant in light of several previous studies. Specifically, Brake and co-workers showed that when a monolayer of phospholipid (containing 2 mol % biotinylated lipid) was formed at an aqueous-LC interface by fusion of vesicles and subsequently exposed to a solution of neutravidin, micrometer-scale domains comprised of segregated proteins and lipids were observed at the LC interface.⁹ This segregation of species led to patterned orientations of the LC. Furthermore, de Tercero *et al.* demonstrated that non-specific interactions of proteins with interfaces of

5CB decorated with partial monolayers of DLPC also led to formation of micrometer-sized, patterned domains of LC, consistent with penetration of the protein into the lipid-laden interface (and segregation of the species at the interface).⁵⁴ In contrast to these past studies, the interaction of vesicles with protein-decorated LC interfaces, as reported in this paper, does not lead to micrometer-scale, lateral segregation of phospholipids and proteins leading to domains of either planar or homeotropic ordering of LCs. Instead, the continuous tilting of the LC reported in this paper suggests a physical picture where phospholipids and proteins form sub-optical domains (see below).

The above-described interpretation of the continuous ordering transition is inspired by previous studies that have reported that heterogeneous interfaces comprised of nanoscopic patches that cause homeotropic or planar anchoring of LCs can give rise to micrometer-scale tilting of the LC.^{28, 55, 56} The pattern of local surface-imposed orientations of the LC becomes homogeneous in the bulk of the LC in order to minimize the elastic energy of the LC (by relaxing the discontinuity in the director field near the alignment layer). In contrast, a mixture of two species, one of which causes homeotropic alignment and the other causes planar alignment, if mixed homogeneously at the molecular level, will not give rise to tilted states of the LC. That is, for a molecularly mixed alignment layer, the change from planar to homeotropic alignment (or vice versa) occurs in a discontinuous manner.^{28, 44} For example, Bos and coworkers demonstrated that a homeotropic alignment layer, when deposited as a continuous film over a first layer that causes planar anchoring of a LC, leads to a discontinuous transition of the orientation of the LC from planar to homeotropic as the thickness of the top layer is increased.⁴⁴ In contrast, when the overlayer causing homeotropic alignment of the LC formed a discontinuous film, a continuous transition in orientation of the LC was observed with increasing coverage of the surface by the top layer.⁴⁴ The observation of a continuous change in the tilt of the LC is, therefore, consistent with an inhomogeneous LC interface comprised of nano-domains of proteins and phospholipids. While additional studies are required to find direct evidence of nanoscopic domains of proteins and phospholipids at the aqueous-LC interface, we note that past studies have reported that surfactant-driven displacement of proteins from an oil-water or air-water interface results in the formation of surfactant and protein-rich domains with sub-micrometer sizes.^{38, 47}

Another significant observation reported in this paper is the dependence of the response of the LC on the state of the proteins adsorbed onto the LC interface. Many past studies have reported that proteins undergo conformational changes upon adsorption to interfaces.⁵⁷ For example, at hydrophobic interfaces, proteins expose their hydrophobic (interior) amino acid residues to maximize interactions of these residues with the hydrophobic interfaces. This restructuring (denaturation) of the protein can promote cross-linking of proteins on the interface and the formation of a cohesive gel-network.⁵⁸ Of particular relevance to our studies, Mackie and co-workers have shown that surfactant-driven displacement of a protein film becomes increasingly difficult with increasing age of the protein film.⁴⁷ The extent of conformational change of the proteins is also affected by the degree of crowding of proteins at interfaces: The lower the interfacial density of proteins, the more space proteins have to spread and unfold to maximize their interactions with the interface.⁵⁰ For example, Norde and co-workers have demonstrated that the α -helix content of serum albumin decreases with decreasing surface coverage.⁵¹ Our observation that LC ordering transitions induced by specific capture of vesicles are influenced by both aging and crowding of proteins at the interface suggests that the conformational state of the proteins at the LC interface is a central factor underlying these LC ordering transitions. We interpret our results to suggest that the LC ordering transitions involve at least two key processes. The first process involves the accumulation of phospholipid near the interfacial region of the LC via specific binding of the vesicles to the protein-decorated interface. The second process is the phospholipid-

driven displacement of the proteins from the LC interface. Protein films that are difficult to displace impede incorporation of lipids into the interface of the LC.⁴⁷

Finally, we note that several studies have reported that biomolecular interactions (enzymatic events⁵⁹, hybridization of DNA³, antibody-antigen interaction⁶⁰) can perturb the ordering of LCs at amphiphile-decorated interfaces. While the molecular mechanisms underlying several of the ordering transitions remain to be fully elucidated, these past examples, when combined with the current study, serve to illustrate the richness of interfacial phenomena that result from the competing influences of proteins and amphiphiles on the ordering of LCs at LC-aqueous interfaces.

Conclusion

A key conclusion of the study reported in this paper is that interfaces between LCs and aqueous solutions, when decorated with proteins, permit specific binding events involving vesicles to be amplified into orientational transitions in the LC. We interpret our results to indicate that the ordering transition reflects the competitive interactions of interfacial proteins and phospholipids with the LCs. Accumulation of vesicles at the LC interface due to specific binding of ligands with receptors pre-adsorbed at the interface facilitates the transfer of phospholipids from the vesicles to the interface of LC, which in turn triggers a continuous ordering transition of the LC to a homeotropic orientation and a distinct change in the optical appearance of the LC. Non-specific interactions of vesicles with protein-decorated LC interfaces were also observed to cause ordering transitions in the LC, but the dynamics of those transitions were significantly slower than that observed in the presence of the specific binding events. Significantly, the response of the streptavidin-decorated LC to vesicle binding was a *continuous* change in the tilt of the LC, hinting at the likely presence of sub-micrometer domains of proteins and phospholipids. Our results also reveal that the LC ordering transition is influenced by aging and crowding of the interfacial proteins. Both effects are consistent with the proposition that the state of the proteins adsorbed onto the LC interface influences the ease of displacement of the proteins from the interface by phospholipids, a process that appears to be necessary for the LC ordering transition to be observed. Finally, the phenomena reported in this paper using the streptavidin-biotin system were observed also when using a LC interface decorated with anti-biotin antibody. Overall, the results presented in this paper suggest that LCs offer the basis of a novel tool for fundamental studies of proteins and amphiphiles at interfaces and, specifically, they offer new methods to report for specific capture of ligand-containing vesicles on protein-decorated interfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Epifluorescence micrographs of an aqueous-5CB interface following incubation against (a) aqueous buffer (0.5 mM PBS) or (b) 1 μM streptavidin in aqueous solution (1 mol % Texas-Red-conjugated streptavidin). Corresponding optical micrographs (crossed polarizers) of the nematic 5CB film are shown in (c) and (d) respectively. (e) Schematic illustration of the director profile of the LC corresponding to the optical images shown in (c) and (d). (f) Plot of epifluorescence intensity of Texas Red-conjugated streptavidin at the aqueous-LC interface after incubation against the indicated concentration of streptavidin in aqueous solution for 90 min (blue diamonds). The red diamond indicates the epifluorescence intensity of a LC interface that was incubated against 1 μM streptavidin for 24 h.

Figure 2.

Ordering transitions induced in films of nematic 5CB by incubation of vesicles ($50\ \mu\text{M}$ of total phospholipid concentration) against unmodified or protein-decorated aqueous interfaces of the LC. (a) Optical images (crossed polarizers) of nematic 5CB with unmodified aqueous interface (no adsorbed proteins) upon incubation against a dispersion of vesicles. (b) Optical images of 5CB with a streptavidin-decorated LC interface upon incubation against a dispersion of DLPC vesicles (no biotin-DOPE). (c) Optical images of 5CB upon incubation of a non-specific IgG-decorated interface of the LC against a dispersion of vesicles containing 5 mol % biotin-DOPE. (d) Optical images of 5CB with a streptavidin-decorated LC interface upon incubation against a dispersion of vesicles containing 5 mol % biotin-DOPE. (e) Tilt angle of 5CB at the aqueous-LC interface, plotted as a function of time following incubation against dispersions of vesicles ((a) – circles; (b) – squares; (c) – triangles; (d) – diamond).

Figure 3.

(a) Schematic illustration of the anchoring of 5CB during fusion of a phospholipid vesicle onto an unmodified aqueous-LC interface. (b) Schematic illustration of a biotinylated vesicle binding to an aqueous-LC interface decorated with a high density of streptavidin, with subsequent transfer of phospholipid onto the LC interface and partial displacement of protein from the interface. (c) Schematic illustration of a biotinylated vesicle binding to an aqueous-LC interface decorated with a low density of streptavidin (or an aged protein layer). The interfacial protein unfolds on the interface, leading to a bound state on the interface that is less readily displaced by phospholipid.

Figure 4.

Plot of (a) epifluorescence intensity of BODIPY-DHPE and (b) phospholipid density, both for a streptavidin-decorated aqueous-5CB interface following incubation against dispersions of vesicles with (5 %) or without (0 %) biotin-DOPE (50 μ M of total phospholipid concentration, 1 mol % BODIPY-DHPE). (c) Plot of loss of epifluorescence intensity of Texas Red-conjugated streptavidin following incubation of a streptavidin-decorated interface of nematic 5CB (1 mol % labeled streptavidin) against dispersions of vesicles with (5%) or without (0%) biotin-DOPE.

Figure 5.

Effect of protein aging on the LC ordering transition triggered by specific binding of biotinylated vesicles to a protein-decorated interface of a LC. (a) Optical images (crossed polarizers) of nematic 5CB upon incubation of a freshly-prepared streptavidin-decorated aqueous-LC interface against biotinylated vesicles (0.1 mM). In (b), the streptavidin-decorated interface was aged overnight prior to incubation against biotinylated vesicles. (c) Tilt angle of 5CB at the aqueous-LC interfaces corresponding to (a) diamonds and (b) squares.

Figure 6.

Influence of the interfacial density of streptavidin on ordering transitions induced in nematic 5CB by specific binding of biotinylated vesicles. (a–e) Optical images (crossed polarizers) of nematic 5CB upon incubation of streptavidin-decorated aqueous-LC interfaces against biotinylated vesicles (0.1 mM, 5 mol % biotin-DOPE). The interfaces were contacted with solutions of streptavidin having concentrations of (a) 10, (b) 1, (c) 0.1, (d) 0.01, and (e) 0.001 μM prior to incubation against the dispersions of biotinylated vesicles. (f) Plot of epifluorescence intensity of Texas Red-conjugated streptavidin (1 mol %) before and after incubation of the LC interfaces against the dispersions of biotinylated vesicles. (g) Plot of the increase in epifluorescence intensity of BODIPY-DHPE upon incubation of streptavidin-decorated LC interfaces against dispersions of vesicles (1 mol % BODIPY-DHPE).

Figure 7. Ordering transitions induced in films of nematic 5CB by specific binding of biotinylated vesicles (0.1 mM) to anti-biotin antibody-decorated aqueous-5CB interfaces. Optical images (crossed polarizers) of nematic 5CB that result from incubation of anti-biotin antibody-decorated interface against dispersions of vesicles containing (a) 5 mol % and (b) 0 mol % biotin-DOPE. (c) Tilt angle of 5CB at the aqueous-LC interfaces corresponding to (a) diamonds and (b) squares.