Cooperative Self-Assembly at Interfaces and its Impact on Long Range Molecular Orientation

By Patrick Noonan

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written by Patrick Scott Noonan

has been approved for the Department of Chemical and Biological Engineering

_____________________________________
Daniel K. Schwartz

_____________________________________
Andrew P. Goodwin

The final copy of this thesis has been examined by the signatories, and we
find that both the content and the form meet acceptable presentation standards
of scholarly work in the above mentioned discipline.
Abstract

Noonan, Patrick Scott (PhD, Chemical and Biological Engineering Department)

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Thesis directed by Professor Daniel K. Schwartz

The prominence of molecular self-assembly in chemical and biochemical processes related to sensing, electro-optical applications, and cellular mechanisms motivates fundamental studies of self-assembly processes. Here, we study how biomolecules and organic compounds interact at interfaces to cooperatively self-assemble into organized structures. In particular we are interested in understanding how liquid crystals (LCs), capable of forming anisotropic phases, behave under varying interfacial conditions. First, we systematically decreased the monolayer coverage of long chain alkylsilanes to study the interplay between surface energy and hydrocarbon chain density. Next, we studied the molecular mechanisms of how nucleic acids influenced interfacial molecular orientation at surfactant laden aqueous/LC interfaces. These studies motivated subsequent exploration of how bio-molecular interactions involving aptamers might induce LC reorientations. Finally, we explored ways to use receptor-mediated liposome fusion to induce LC reorientations at the aqueous/LC interface.

The findings presented here elucidate the molecular mechanisms that dictate LC reorientations capable of signal transduction in molecular sensing. For example, studies at the solid/LC and aqueous/LC interface revealed that a relatively low sub-monolayer coverage (~11%) of long alkyl chains can induce homeotropic alignment of calamitic LCs. Furthermore, when hydrophobic polyanions adsorb to aqueous/LC interfaces with an alkyl chain coverage close to this
threshold, a LC reorientation to a planar/tilted LC alignment occurred. Experiments revealed that the interaction between exposed hydrophobic moieties and the LC were of critical importance toward inducing this LC reorientation. Furthermore, when specific binding events (i.e. DNA hybridization, aptamer-ligand binding) were used to modulate the hydrophobic exposure of biomolecules at LC interfaces, the LC alignment was correlated with the hydrophobic exposure. Finally, we designed and characterized aqueous/LC interfaces that inhibited the spontaneous fusion of liposomes and the non-specific adsorption of macromolecular proteins, enabling receptor-mediated (i.e. DNA hybridization) liposome fusion. These studies advance the understanding of molecular mechanisms that dictate LC alignment and provide examples of how liquid crystal reorientations can be exploited for molecular bio-sensing. Thus we have used a range of organic and bio-molecular species to gain a better understanding of the self-assembly processes that dictate LC alignment at solid/LC and aqueous/LC interfaces.
This work is dedicated to my parents, Scott and Jane Noonan,
for their unrelenting support and undeniable wisdom.

“Quick but not rushed”
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Chapter 1: Introduction

Molecular self-assembly processes involve the spontaneous formation of organized structures or aggregates driven by non-covalent interactions. In some cases native chemical properties allow enthalpic driving forces (e.g. hydrogen bonding, Van der Waals) to overcome the entropic penalty associated with order. In other cases non-intuitive entropic driving forces (e.g. excluded volume, depletion attraction) induce the formation of organized structures. Cooperative self-assembly is when multiple molecular species work together to initiate assembly. For example, when two solutes of different sizes are dispersed in solution, the larger solutes aggregate, effectively reducing the excluded volume associated with their hydrodynamic radius. This reduction in the excluded volume increases the translational entropy of the smaller solutes enough to result in an overall increase in the entropy of the system. In the absence of small solutes the larger solutes do not aggregate, thus the large and small solutes act cooperatively to induce aggregation via self-assembly.

In this dissertation, several examples of cooperative self-assembly at anisotropic interfaces are presented that involve liquid crystals (LC), organic monolayers, nucleic acids, proteins, and colloidal assemblies. A key aim of the work presented here was to demonstrate how LCs can be used to transduce nucleic acid binding events toward molecular bio-sensing applications. LCs are an anisotropic self-assembling substrate that we exploited to study how biomolecules (e.g. nucleic acids, proteins, phospholipids) affect self-assembly at LC interfaces. An introduction to LC fundamentals is first presented. Then, a discussion of biosensor technologies provides motivation for the proposed studies. The proceeding chapters outline experimental details and findings from studies that probed how thin films and adsorbates affected the interfacial environment associated
with LC self-assembly, discuss how these findings constitute a significant contribution to the scientific community, and propose future work.

### 1.1 Liquid crystal order

Ordered systems arise from intermolecular forces that that overcome the entropic penalty associated with order. For example, solid crystals often employ covalent linkages to arrange their molecules in ordered patterns that extend to three dimensions. Conversely, the lack of strong interactions in fluids may result in the absence of molecular order. Liquid crystals are a state of matter that is intermediate between solid crystals and isotropic fluids since they have 0-2 dimensions of translational order and also exhibit orientational order. Molecular order is achieved in liquid crystal phases through self-assembly pathways. For example, the packing of rod-like

![Figure 1.1 – Order in calamitic LCs:](image)

- **A** LC molecules are oriented with their long axis parallel to the director (n) (S< 1.0).
- **B** Slight deviations from the director (n) occur but an average orientation is maintained and $S > 0$.
- **C** In the isotropic phase no orientational order occurs ($S = 0$).
molecules results in excluded volume effects that induce a coordinated molecular orientation. If two rod-like molecules pack perpendicular to each other, the excluded volume is greater than if they pack parallel to each other. Thus in order to maximize the orientational entropy associated with packing, they orient themselves in a coordinated direction. Other driving forces capable of inducing order in liquid crystal phases involve induced dipole-dipole interactions or aromatic stacking which stabilizes orientational, positional or translational ordering. The extent of orientational order in liquid crystals is defined by the order parameter, $S = \langle (3\cos^2\theta - 1)/2 \rangle$, where $\theta$ is the local angle between the molecular axis and the preferred LC orientation (i.e. the director, $n$). Thus in a completely ordered system $S$ equals unity while in an isotropic system $S$ is equal to zero (Figure 1.1C). Typical order parameters for liquid crystal materials range from 0.4-0.7 (e.g. Figure 1.1A,B).

### 1.2 Liquid crystal mesophases

Since the discovery of LCs in 1888, several LC mesophases have been discovered from a variety of molecular species including rod-shaped molecules, lipid aggregates, and bent-core molecules. Liquid crystal mesophases depend on either temperature (i.e. thermotropic), or concentration (i.e. lyotropic). For example certain surfactant dispersions, at high enough concentrations, aggregate into columnar structures that self-assemble into ordered phases. However, at low concentration (e.g. <CMC) these systems are primarily composed of monomers and are therefore isotropic. Thermotropic liquid crystals transition between mesophases at specific temperatures. Examples include smectic A, smectic C, and nematic. (Figure 1.2) Nematic liquid crystals have no translational or positional ordering, but do have orientational order. Smectic phases have translational order in one dimension, where the average spacing between molecules is constant along a common axis.
smectic A (SmA) phases the orientation of the long molecular axis is parallel to the axis associated with the positional order while in Smectic C (SmC) the molecular axis is tilted relative to this axis. Another example involves the chiral liquid crystal phase smectic C* (e.g. SmC*). In the SmC* phase the orientation of molecules between smectic layers rotates in a helical pattern. In a thermotropic liquid crystal, increasing order (e.g. Isotropic → Nematic → Smectic → Crystalline) correlates with decreasing temperature. These liquid crystal phases lead to a range of interesting material properties (e.g. birefringence\(^3\), ferroelectricity\(^5\)) that have been exploited for a range of technological applications. Here, our focus is on how we can utilize the optical properties associated with thermotropic nematic liquid crystals for molecular bio-sensing.

Figure 1.2 – *Liquid Crystal Mesophases*: In nematic liquid crystals the phases will transition from left to right with increasing temperature (thermotropic) or concentration (lyotropic). (note: ordered phases are drawn with high order parameters for simplicity, however in real LC samples S=0.4-0.7)
1.3 Birefringence and Polarized Light

Birefringent materials characteristically induce the double refraction of incident light to create ordinary and extraordinary rays defined by their associated refractive indices ($n_o$ and $n_e$, respectively).\(^6\) Importantly, these rays are polarized in planes perpendicular to each other. Thus, when linearly polarized light encounters a birefringent material the transmitted wave effectively becomes elliptically or circularly polarized. The subsequent transmission of this elliptically polarized light through a polarizer aligned normal to the incident polarization (i.e. the analyzer) transmits only the components of the elliptically polarized light that are parallel to the polarization associated with the analyzer. The resultant light has a wavelength generated by constructive and/or destructive

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Figure 1.3 – Anisotropy in LCs: (A) Molecular structures of the components that make up the LC mixture E7. (B) A schematic illustration of anisotropy in LCs [note: for simplicity the schematic depicts near perfect order with $S=1$ while in reality LC phases are less ordered with $S=0.4-0.7$] (C) A schematic illustration of a hybrid aligned nematic cell with tilted alignment at the top surface and homeotropic alignment at the bottom surface.
interference due to the phase shift between the ordinary and extraordinary rays. The extent of interference that occurs depends on birefringence, \( B = |n_e - n_o| \), and material thickness. The wavelengths (i.e. colors) associated with a particular birefringence and material thickness are thoroughly characterized and tabulated. The Michel-Levy chart is a graphical representation of these tabulated values and is often used to determine the birefringence of a material when the sample thickness and wavelength can be measured.

Figure 1.4 – Polarized Light Microscopy: When randomly polarized light passes through a polarizer (P) it becomes linearly polarized. The subsequent transmission through a LC cell with (i) hybrid or (ii) homeotropic alignment results in (i) double refraction or (ii) no change in polarization. When this light subsequently passes through an analyzer (A) either (i) light transmission or (ii) no light transmission is observed.
In thermotropic nematic liquid crystals, the effective birefringence depends on the orientation of the average molecular axis relative to the direction of light propagation. Liquid crystals are optically anisotropic, thus the optical properties of liquid crystals depend on the direction that light propagates through the material. For example a commonly employed liquid crystal is 5CB (i.e. 4-Cyano-4'-pentylbiphenyl, Figure 1.3A). Depending on the direction of light propagation through 5CB (e.g. Figure 1.3B) either the long or short axis (or some intermediate between the two) is encountered. The ordinary refractive index is measured by light polarized perpendicular to the optical axis (or long axis) \((n_{\perp})\), while the extraordinary refractive index is measured by light polarized parallel to the optical axis \((n_{||})\). When the propagation of light is at a non-zero angle relative to the optical axis \((\theta)\), an effective value \((n_{\text{eff}})\) for the extraordinary refractive index must be calculated\(^8\) that depends on the angle \(\theta\):

\[
n_{\text{eff}}^2 = \frac{n_{||}^2 n_{\perp}^2}{n_{||}^2 \cos^2 \theta + n_{\perp}^2 \sin^2 \theta} \quad \text{(eq. 1.1)}
\]

Thus, the effective birefringence in this case is defined as \(\Delta n_{\text{eff}} = n_{\text{eff}} - n_o = n_{\text{eff}} - n_{\perp}\). In a hybrid aligned nematic liquid crystal cell (Figure 1.3C) with a defined alignment \((\theta_1)\) at one interface and a different alignment \((\theta_2)\) at the opposing interface, the effective birefringence can be calculated by integrating along the \(z\)-direction of the LC cell:

\[
\Delta n_{\text{eff}} = \frac{1}{\theta_1 - \theta_2} \int_{\theta_2}^{\theta_1} \frac{n_{||} n_{\perp} d\theta}{(n_{||}^2 \cos^2 \theta + n_{\perp}^2 \sin^2 \theta)^{1/2}} - n_{\perp} \quad \text{(eq. 1.2)}
\]

Using this equation, one can calculate \(\theta_2\) in a hybrid aligned nematic cell, if \(\theta_1\) is known (e.g. strong homeotropic alignment \(\theta_1 = 0\)) and the cell thickness and birefringence can be measured.
In general, polarized light is qualitatively used to distinguish between LC orientations with the molecular axis parallel (homeotropic), or tilted relative to the plane of incident light. Homeotropically aligned LCs have an effective birefringence that approaches zero, thus extinction occurs when a homeotropically aligned LC cell is viewed between crossed polarizers (Figure 1.4ii), and a qualitatively dark image is observed. Conversely, LC orientation that is tilted or planar yields a non-zero effective birefringence that depends on LC orientation. (Figure 1.4i) Simply stated, when viewing a nematic liquid crystal cell between crossed polarizers, homeotropic alignment across the entire z-axis reveals a qualitatively dark image while tilted or planar orientation at one or both interfaces reveals a bright image with interference colors that depend on birefringence. (Figure 1.4) Thus, polarized light allows one to distinguish between these two states without employing the quantitative analysis described above. These unique optical properties have historically inspired the investigation of ways to control liquid crystal orientation for display and optoelectronic applications and more recently for sensing applications.

![Figure 1.5 – Schematic representation of the zenithial and azimuthal angle in relation to a single LC molecule](image-url)
1.4 Elastic and Surface Anchoring Energy of Liquid Crystals

A characteristic property of liquid crystals is the elastic energy associated with coordinated molecular packing. In the idealized case without defects or boundaries that induce distortions to LC orientation, the LC director aligns in a uniform direction. However, when distortions are present, as in hybrid aligned nematic (HAN) LC cells (Figure 1.3C), the resulting director field must balance the elastic and surface free energies. The elastic energy is defined by splay, twist and bend distortions. This elastic energy is thoroughly characterized and is often calculated using the Frank elastic energy:

\[
F_{\text{elastic}} = \frac{1}{2} [K_s(\nabla \cdot n)^2 + K_t(n \cdot \nabla \times n)^2 + K_b(n \times \nabla \times n)^2] \quad (\text{eq. 1.3})
\]

where \(K_s\), \(K_t\), and \(K_b\) respectively represent the splay, twist and bend elastic constants. These constants have been measured for a number of liquid crystal materials to allow for the calculation of director profiles. Often, these constants are on the same order of magnitude (e.g. 5CB: \(K_s=7.0\text{pN}, K_t=4.6\text{pN}, \) and \(K_b=10.4\text{pN}\)) allowing for the Frank elastic energy to be approximated using a one constant approach:

\[
F_{\text{elastic}} = \frac{1}{2} K [ (\nabla \cdot n)^2 + (\nabla \times n)^2 ] \quad (\text{eq. 1.4})
\]

In most applications that use LCs it is often critical to define LC orientation (e.g. homeotropic or planar) at one or more interface. The driving forces for LC alignment may involve purely physical effects that minimize distortions in the director field or intermolecular forces such as coordination interactions, excluded volume, and cooperative self-assembly. The orientation in a nematic LC is defined as zenithial (relative to the z-axis) or azimuthal (relative to the x-axis) (Figure 1.5). It follows that LC alignment layers define a preferred zenithial (\(\theta_0\)) and azimuthal (\(\phi_0\)) alignment, called the
easy axis orientation. Furthermore the strength of the easy axis alignment is quantified by a surface anchoring energy. A function for the overall surface free energy ($f_s$) in nematic LCs is commonly defined according to the Rapini and Papoular model as:

\[
f_s = \frac{1}{2} [W_p \sin^2(\theta - \theta_0) + W_a \sin^2(\phi - \phi_0)]
\]  

(eq. 1.5)

where $W_p$ and $W_a$ are the polar and azimuthal anchoring coefficients, respectively. These anchoring coefficients can be calculated by measuring the deviation of the LC director ($\theta, \phi$) from the easy axis ($\theta_0, \phi_0$). In general, strong anchoring is associated with anchoring coefficients on the order of $\sim 10^{-4}$ J/m$^2$ while weak anchoring is associated with values less than $\sim 10^{-7}$ J/m$^2$. When calculating LC director fields (in the absence of electric and magnetic field distortions), the surface free energy is added to the Frank elastic energy described above so the overall free energy can be minimized.

1.5 Liquid Crystal Alignment Layers

Several strategies for fabricating LC alignment layers utilize micro-patterned surfaces, or organic and inorganic thin films. Zenithial liquid crystal orientation normal to the z-axis (planar orientation) is often achieved using polymer coatings formulated to induce strong planar anchoring (e.g. nylon, polyimide). Furthermore, when these substrates are mechanically rubbed in a uniform direction, they may induce azimuthal alignment of the director. Mechanical rubbing introduces micro-groves into the alignment layer and orients the average molecular axis parallel to the micro-groves in order to minimize distortions in the director field. Another commonly desired LC orientation involves zenithal alignment parallel with the z-axis (i.e. homeotropic). One way to achieve this type of alignment involves using surfaces functionalized with long chain hydrocarbons. In this case, calamatic LC molecules intercalate between long alkyl chains and cooperatively assemble into a configuration with alkyl chains and the long axis of the LC molecules
aligned normal to the surface plane. Furthermore, studies at aqueous/LC interfaces revealed that certain surfactants could be used to induce homeotropic alignment.\textsuperscript{15, 16} Importantly, only long chain surfactants that linearly penetrated the LC induced homeotropic orientation. When the alkyl chain was below a particular threshold length or bolaform surfactants were used, homeotropic orientation was not observed. Interestingly, the charge of the surfactant head group did not generally affect the ability for surfactants to induce homeotropic orientation. Thus, a range of surfactant molecules including cationic\textsuperscript{15}, anionic\textsuperscript{16}, non-ionic\textsuperscript{15}, fatty acids\textsuperscript{17}, and phospholipids\textsuperscript{18} are known to induce homeotropic orientation when deposited at high enough concentrations to aqueous/LC interfaces.

1.6 Adsorbates at Liquid Crystal Interfaces

More recent studies revealed that certain molecular species or moieties disrupted the interfacial structures that dictated particular LC orientations, including long alkyl chain alignment layers that induced homeotropic anchoring. For example, when proteins (e.g. bovine serum albumin) adsorbed to substrates modified with long chain hydrocarbons (e.g. Dimethylloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride : DMOAP), they systematically reduced the LC pre-tilt angle with increasing concentration.\textsuperscript{19} It was speculated that the non-specific adsorption of proteins blocked the intercalation of calamatic LC molecules between hydrocarbon chains at the interface\textsuperscript{14}, preventing homeotropic orientation. Another example involved mixtures of PEG modified lipids (PEG-lipid) and saturated phospholipids (e.g. DPPC).\textsuperscript{20} Increasing the PEG-lipid:DPPC ratio induced the formation of PEG-lipid rich and PEG-lipid lean domains. PEG-lipid lean domains caused homeotropic orientation, while PEG-lipid rich domains were correlated with planar or tilted LC orientation. Interestingly, the planar/tilted LC orientation associated with PEG-lipid lean domains
occurred despite a relatively high coverage of long hydrocarbon chains. It was speculated that PEG disrupted the packing of hydrocarbon chains in a way that inhibited their ability to induce homeotropic orientation. These examples illustrate how LC reorientations were induced by molecular species or moieties that non-specifically disrupted interfacial structure.

More specific control over LC orientations at aqueous interfaces has been achieved by exploring a range of bio-molecular interactions\textsuperscript{21}. For example, the hydrolysis of phospholipid monolayers by the enzyme phospholipase A has been shown to induce LC reorientations from homeotropic to planar.\textsuperscript{22} Furthermore, studies have revealed that specific protein binding events can mediate the deposition of lipids, and a subsequent transition to homeotropic orientation, at aqueous/LC interfaces.\textsuperscript{23} Another example exploited oligopeptide decorated interfaces where the structure of the oligopeptide monolayer translated to the orientation of the adjacent LC interface.\textsuperscript{24} In this example, oligopeptides were covalently attached to surface anchored lipids. When the appropriate protease was added, the oligopeptide organization was disrupted and a LC reorientation occurred. These examples demonstrate how LC reorientations were induced by specific binding events and illustrate the potential for sensing applications that use LCs as a transduction element.

The majority of previous studies that employed specific binding events to induce LC reorientations focused primarily on protein binding events\textsuperscript{21}, while a smaller fraction studied binding events involving nucleic acids\textsuperscript{25-28}. The studies presented in this dissertation involve DNA-DNA or DNA-ligand binding events that occur at or adjacent to liquid crystal interfaces. For example, nucleic acids have been shown to be capable of disrupting the interfacial structures associated with the surfactant induced homeotropic orientation discussed above.\textsuperscript{26} When ssDNA was adsorbed to a
cationic surfactant-laden aqueous-LC interface, a LC anchoring transition from homeotropic to planar occurred. When the ssDNA probe subsequently hybridized with a complementary strand, a transition back to homeotropic LC orientation took place. The original work describing this phenomenon was the first example where liquid crystals transduced DNA hybridization into a measurable signal and motivated much of the work reported here. In particular, we sought to understand the molecular mechanisms dictating the LC reorientation that occurred upon ssDNA adsorption and subsequent hybridization. By doing so, we hoped to extend this approach for detecting binding events involving aptamers. Thus we used a balanced approach that involved studying the fundamental mechanisms that dictate self-assembly processes at liquid crystal interfaces toward developing advanced biosensors using nucleic acids as our probe species.

1.7 Biosensors

A sensor detects and/or measures a property so that it can be monitored or recorded. Sensors are generally categorized as physical, chemical, or biological. Physical sensors measure physical quantities (e.g. weight, distance, etc.), chemical sensors detect the presence of chemical species, and biological sensors are a subset of chemical sensors where the detection element is biological in nature. All sensors have three universal components: a detector, transducer, and measuring device. The detection element recognizes an analyte of interest, the transducer translates this recognition event into a measurable response, and the measuring device converts this response into a measurable signal. This dissertation aims to better understand the capabilities of bio-sensors that exploit nucleic acid binding events as a detector, liquid crystals as a transducer, and polarized light as a measuring device.
Sensor performance is defined by several properties including selectivity, sensitivity, dynamic range, accuracy, operating range, response time, recovery time, cost, and working lifetime. An important challenge in bio-sensor research is to design a sensor that can universally be used to simultaneously detect and quantify a multitude of chemical species (i.e. multiplexed) under a wide range of operating conditions. However, improvement in one area often leads to deficiencies in others (e.g. high sensitivity leads to poor selectivity or dynamic range). Thus, there is significant interest in designing and understanding sensor schemes that maximize performance in at least one of these areas (e.g. high sensitivity, multiplexed, wide operating range). Binding events involving nucleic acids have proven valuable as a detection element capable of multiplexed detection with high selectivity.30

1.7.1 Nucleic Acid Probes

Since the discovery of the structural foundation of DNA in 195331, nucleic acids have become a valuable tool for biotechnological applications.32 The correlation of genomic DNA sequences with disease states, pathogens, and cellular function has led to significant advances in clinical and molecular diagnostics. Thus, extensive work is ongoing toward developing advanced strategies for detecting DNA hybridization events and a detailed discussion of these technologies is provided in later chapters. Recently, nucleic acids have demonstrated an even greater capacity for detecting a multitude of molecular species. In particular, it was shown that nucleic acids not only bind to complementary sequences with high sensitivity and specificity, but also to certain ligands. The RNA world theory proposes that ribo-nucleic acids were the precursor for life as we know it today.33 In this theory RNA is capable of catalyzing reactions between simple chemical species, complex peptide bonds, and intricate protein folding mechanisms. It follows that nucleic acids have a built in
functionality for recognizing molecular species other than complementary nucleic acids. This theory, in part, led to the discovery of functional nucleic acid molecules called aptamers.\textsuperscript{34, 35} Aptamers are nucleic acids that have been synthetically evolved through the SELEX (systematic evolution of ligands by exponential enrichment) process to bind to specific ligands (e.g. ions, small molecules, proteins)\textsuperscript{36}. Aptamers are capable of molecular recognition that is comparable, if not better, than monoclonal antibodies. Thus, bio-sensing strategies that once were limited to detecting DNA hybridization are now being exploited for detecting aptamer-ligand binding events, making nucleic acids an even more useful probe then previously imagined.

1.7.2 Signal Transduction with Liquid Crystals

The objective of the work presented in this dissertation is to exploit the unique optical properties of liquid crystals toward advanced bio-sensing strategies. As discussed above, polarized light offers a simple way to measure molecular LC orientations. LC based sensors operate by using polarized light to measure perturbations in LC orientation induced by the presence of specific analytes. When properly employed LCs offer a signal transduction approach that is label-free, portable, inexpensive, observable by the naked eye, multiplexed, and naturally amplified. Thus, LC transduction elements address some of the key challenges associated with developing a universal bio-sensor strategy such as operating conditions, cost, and sensitivity. These advantages provide the technological motivation for using liquid crystals in advanced bio-sensing strategies and are discussed in greater detail throughout this dissertation.
It is postulated that by studying the fundamental mechanisms that dictate LC alignment at interfaces, improved strategies for using LCs to transduce bio-molecular interactions can be achieved. The proceeding chapters present novel findings that support this premise.

1.8 References


Surfaces functionalized with a self-assembled monolayer (SAM) formed from a mixture of two alkylsilanes with different chain lengths have been designed to simultaneously improve the liquid crystal (LC) wettability and promote homeotropic anchoring of the LC. Most chemically functionalized surfaces (e.g. long alkyl chain SAMs) that promote homeotropic alignment of LC possess low surface energy and result in poor LC wettability, inhibiting LC infiltration into microstructured surfaces, and sometimes resulting in LC dewetting from the surface. However, a surface modified with a mixed SAM of octadecyltriethoxysilane (C18) and ethyltriethoxysilane (C2) exhibited very low LC contact angle while providing homeotropic anchoring. Ellipsometry was used to correlate the bulk concentration of C18 in the deposition solution to the surface coverage of C18 in the mixed monolayer; these bulk and surface concentrations were found to be equal within experimental uncertainty. The LC contact angle was found to depend non-monotonically with the surface coverage density, with a minimum (14.4 ± 0.1°) at a C18 surface coverage of 0.26 ± 0.08. Homeotropic LC anchoring was achieved at a C18 surface coverage of ≥ 0.11 ± 0.04, in the regime where a minima in the LC contact angle was observed. The practical application of this approach to surface modification was demonstrated using a micropillar array sensor substrate. When the array was functionalized with a conventional C18 SAM, the LC did not infiltrate the array and exhibited a contact angle of 47.4 ± 0.5°. However, the LC material successfully infiltrated and wetted the same micro-structured substrate when functionalized with a C18/C2 mixed SAM, while still exhibiting the desired homeotropic anchoring.
2.1 Introduction

Liquid crystal (LC) - based sensors show promise in many applications, including the detection of infectious disease biomarkers, environmental pollutants, harmful gases, and protein reactions. For LC sensors to function properly, a number of critical parameters must be carefully controlled, including the LC film thickness, the LC orientation, and substrate surface energy. In typical LC display applications, the LC film thickness is determined by the spacing between two solid surfaces. In sensors, however, the LC film is often supported on a single substrate and is in contact with a vapor or liquid phase that contains the target or analyte species. Therefore, the LC film thickness is more difficult to control. In many cases, this control is achieved by containing the LC within a microstructured environment where capillary interactions can be exploited to maintain a uniform and stable film. A simple example of this, appropriate for prototype applications, involves an electron microscopy grid. The holes within such a grid can be filled with LC, and to a first approximation the LC layers adopts a thickness equal to the height of the grid. A more robust and reproducible approach involves stabilizing a continuous LC film in a microstructured environment, e.g. an array of cylindrical micro-pillars on a glass substrate. In this scenario, an LC material infiltrates the entire array, filling the space between the micro-pillars, and capillary forces stabilize the LC film at a thickness equal to the height of the micro-pillars.

In virtually all LC applications, a specific LC orientation must be defined at one or more interfaces. Examples include homeotropic anchoring, where the long axes of the LC molecules are oriented in the surface normal direction, or planar anchoring, where the LC long axes lie in the surface plane. A number of standard surface modification approaches have been developed and are used to control LC surface anchoring on planar surfaces for various applications. Microstructured substrates
used for sensor applications need to fulfill an additional requirement, however, because the LC material must infiltrate the substrate, maintain a uniform thickness, and remain stable. In particular, if the balance of interfacial free energies is such that the contact angle of the LC on the alignment layer is too large, the LC material may not spontaneously infiltrate the microstructured environment or it may de-wet during use. Thus, there is a need for chemical functionalization technologies that can be used to modify the surfaces of microstructured environments and that spontaneously promote LC wetting of these environments.

It is often desirable to maintain homeotropic LC orientation at a solid-LC interface for sensor applications.\textsuperscript{3,7,8,15} On a conventional planar glass surface, this is typically achieved by using surfaces functionalized with a self assembled monolayer (SAM) composed of long chain alkylsilanes.\textsuperscript{11,13} The deposition of an alkylsilane SAM involves spontaneous adsorption from a liquid or vapor phase and an assembly driven by island growth\textsuperscript{16,17}, that is stabilized by covalent Si-O-Si linkages among silane moieties as well as between silane moieties and surface silanol groups\textsuperscript{18,19}. These SAM alignment layers are convenient and appropriate for many LC sensor substrates. However, surfaces functionalized with long-chain alkylsilane SAMs possess low surface energy and LC materials (and hydrocarbon liquids in general) typically exhibit moderately high contact angles making these surfaces inappropriate to support a uniform LC film.

The relationship between LC alignment and the wettability of various monolayer compositions has been extensively studied.\textsuperscript{10,11,20-22} Historically, a relationship was proposed that allowed one to predict the orientation of liquid crystals on a given substrate based on the governing relationship that homeotropic LC orientation can be achieved only on substrates where the LC surface tension
(γ_{LC}) is greater than a critical surface tension of the substrate (γ_{C}) (i.e. high contact angle).^{11,22} Such a relationship suggests that it would be difficult, if not impossible, to design surfaces that are both wettable by the LC and also induce homeotropic anchoring. Despite reports that directly confirm this relationship^{22}, others have shown that it is not universal.^{20,21} Exceptions to the “rule” often involve situations where strong local directional interactions are dominant. For example, carboxylic acid terminated SAMs functionalized with certain metal salts promotes homeotropic orientation and a low LC contact angle of nitrile-containing LCs.\(^9,23\) In this case, the homeotropic orientation is attributed to a specific coordination interaction between the nitrile group of LC and the metal center. Another example involves glass surfaces functionalized with amine-terminated SAMs. Despite the fact that they exhibit increased γ_{C} over other substrates (e.g. functionalized with long chain alkyl silanes)^{24} typically used for homeotropic alignment, amine-terminated SAMs are used to induce homeotropic LC alignment;\(^6,12,14,15\) apparently due to strong localized dipole-dipole interactions between the polar LC molecules and the surface amine.\(^12\) Low LC contact angles have not been reported, however, on amine-terminated glass surfaces.

To date, these specialized examples of LC alignment layers that induce high homeotropic anchoring energy and a low LC contact angle have only been prepared via thiol chemistry on metal surfaces. We were unable to find a published example of a surface functionalization strategy that can be applied to an oxide material, using silane chemistry. In fact, most high energy surfaces possessing low LC contact angles have been found to yield low pretilt angles\(^{10,11,25,26}\) i.e. promote planar or tilted LC alignment.
LC alignment on oxide surfaces functionalized with silanes is of significant technological importance, not only for LC sensor applications but also in the display industry. The utilization of functionalized oxides (e.g. glass, indium tin oxide) allows for improved transmittance over metal surfaces (e.g. gold), which is relevant in the design of LC displays. Metal substrates also require additional processing steps to deposit a thin metal film onto a supporting substrate\textsuperscript{5,23}, while oxide substrates can be functionalized using simple deposition procedures\textsuperscript{13}. Furthermore, LC sensor applications may be sensitive to the presence of ions\textsuperscript{3} and the use of an oxide substrate, over a metallic substrate, can reduce the risk of ionic contamination affecting the sensor response. Therefore, our aim was to develop a SAM that promotes homeotropic anchoring and LC wetting on a functionalized oxide substrate via silane chemistry.

Studies have suggested that homeotropic anchoring can sometimes be achieved by less than complete coverage of long alkyl chains\textsuperscript{3,27,28} We postulated, therefore, that high energy (i.e. LC wettable) surfaces, promoting homeotropic alignment, might be achieved by functionalizing surfaces with mixed SAMs where the surface density of long chains was diminished via mixing with a second component that would serve to reduce the LC contact angle. This strategy allows for the design of SAMs with simple surface chemistry to achieve tunable surface properties. In this report, results from a systematic study of various mixtures of long and short chain alkylsilanes are presented.
2.2 Materials and Methods

2.2.1 Mixed Monolayer Deposition

Self assembled monolayers of alkylsilanes were prepared according to published procedures modified for mixed monolayers. Soda lime glass microscope slides (Corning Inc.) were cleaned sequentially with 2% aqueous micro-90, deionized water (18.2 MΩ), and piranha solution [30% aqueous H₂O₂ (Fisher Scientific) and concentrated H₂SO₄ (Fisher Scientific) 1:3, v/v] at 80°C for 1 hr. (Warning: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store in closed container). After piranha cleaning, the microscope slides were rinsed with deionized water and dried under a stream of ultrapure N₂. A deposition solution of n-butylamine (Fisher Scientific) and alkylsilanes was prepared in toluene (Fisher Scientific) at 1:3:200 volumetric ratio, respectively. The alkylsilanes, octadecyltriethoxysilane (C18), n-butytriethoxysilane (C4), ethyltriethoxysilane (C2), or n-methyltriethoxysilane (C1) (Gelest Inc.), were mixed at varying volumetric fractions \( \frac{v_{C18}}{v_{C(1,2, or 4)} + v_{C18}} \) = 0, 0.06, 0.11, 0.22, 0.33, 0.44, 0.66, 0.88, 1.00. The deposition solution was warmed to 60°C, clean and dry microscope slides were first rinsed with toluene, and then submerged in the warm deposition solution. The slides were incubated in the deposition solution for 1 hour at 60°C. Upon removal, the slides were rinsed with toluene, dried under a stream of ultrapure N₂, and stored at room temperature in a vacuum desiccator.

2.2.2 Contact Angle Measurements

Contact angles (θₐ) were measured on three substrates at three random locations, for each mole fraction of C18 tested, using the static sessile drop method with a custom-built contact angle goniometer. The variability between samples was found not to be significantly different from the
variability within a given sample and the error in the contact angle is reported as the standard error. Water contact angles were measured by adding a ~1μL drop of water to the surface dispensed from a syringe positioned directly above the surface. The drop was imaged immediately, to prevent evaporation, and imaging software (First Ten Angstroms, Inc) was used to extract the contact angle for θC>15°. For θC<15°, ImageJ (NIH Freeware) was used to manually determine the contact angle due to poor fits from the image analysis software at these low contact angles. A LC mixture, E7 (a mixture of three cyanobiphenyls and a cyanoterphenyl, Merck KGaA), was used to measure the LC contact angle and a hydrocarbon oil viscosity standard, N26 (a mixture of poly-alpha olefins, Cannon Instrument Co.), was used to measure the contact angle of an isotropic oil. A micro-pipette (Eppendorf) was used to add a 0.2–0.5μL drop of E7 or N26 onto the surface. The drop was imaged within 10 seconds of contact with the surface and the contact angle was determined using imaging software (θC>15°) or ImageJ (θC<15°).

2.2.3 Polarized Light Microscopy

LC orientations on surfaces functionalized with different SAM compositions were determined by observing LC optical cells using an Olympus microscope (model BH2-UMA) modified for transmission mode. LC cells were prepared by pairing two identically functionalized surfaces facing each other to form a ~5-10 μm thick cavity between them. The cavity was then filled with E7, via capillary action, in between two SAMs prepared from the same deposition solution. LC orientations were determined by imaging the optical cells between crossed polarizers. Homeotropic orientation was characterized by the lack of light transmission upon a complete 360° rotation of the sample, while birefringence due to tilted or planar LC orientation resulted in light transmission when the optical cell was imaged between crossed polarizers.
A micro-structured substrate consisting of a hexagonal array (10 µm edge to edge spacing) of 5µm tall cylindrical polymer micro-pillars (diameter D=10µm) on aluminum silicate glass was used to test the ability of E7 to spread on a micro-pillar array. While the use of different glass substrates for silane deposition has the potential to lead to a varying degree of SAM quality due to differences in the coverage of exposed hydroxyls, the use of appropriate sample preparation procedures (ie. piranha, UV-ozone) results in a similar coverage of exposed hydroxyls and therefore SAM quality. SAMs were deposited onto two different samples of the micro-pillar array using two different deposition solutions \( (v_{C18}/(v_{C2} + v_{C18})=1.0, 0.44) \), following a 1 hour UV-ozone exposure. A 0.2µL drop of E7 was added at the edge of the micro-pillars array and a combination of contact angle goniometry and polarized light microscopy was used to determine if the LC infiltrated the micro-pillar array, and to characterize the LC anchoring if infiltration was successful.
2.2.4 Ellipsometry

A variable angle spectroscopic ellipsometer (J. A. Woollam Co., Inc.) was used to measure the thickness of the SAMs under study. A mixed monolayer (C2/C18) was deposited onto silicon (100) wafers (WRS Materials) at varying ratios according to a procedure modified from that described above. The thickness of native SiO\textsubscript{2} (oxide) layer present on the silicon wafer was measured directly after the piranha cleaning step for each sample. Due to the time required for measurement of the oxide layer there was a lag time between piranha cleaning and monolayer deposition of about two hours, which can result in some contamination of the silicon wafers. To reduce the risk of

![Graph](image)

Figure 2.1 – Mixed Monolayer Film Thickness: (a) Ellipsometric thickness of a C2/C18 mixed monolayer with varying \( \chi_{C18} \); (b) Calculated C18 surface coverage with varying \( \chi_{C18} \). The symbols represent experimental data and the lines represent a fit to models described in the text.
contamination affecting the SAM deposition, the substrates were cleaned prior to SAM deposition using a 1 hour UV-ozone exposure. For all measurements the ellipsometry model assumed a 1mm thick Si layer \((n = 3.875 - 0.023i)\) and the change in phase \((\Delta)\) and amplitude \((\Psi)\) at varying angles of incidence \((60-80^\circ, 5^\circ \text{ increments})\) and wavelengths \((500-900\text{nm}, 100\text{nm increments})\) was measured to calculate the thickness of oxide and SAM assuming equal refractive indices \((n = 1.46)\) for the SAM layer and the oxide layer. An average apparent oxide layer thickness taken across all the samples tested was measured \((14.6 \pm 0.4 \text{ Å})\) and was subtracted from measurements of the combined SAM and oxide layer to determine the SAM layer thickness. We note that contamination of the “clean” surfaces can result in an overestimate of the apparent silicon oxide layer thickness, affecting the absolute values of the SAM thickness. The relative thickness values of different SAMs, however, are relatively insensitive to these concerns. Uncertainties due to fitting of the ellipsometric model were consistently less than the experimental error due to variability within each sample and the error in the film thickness is reported as the standard error due to variability between measurements \((n=3)\).

2.2.5 Determination of Surface Fractions

The monolayer thickness is known to be very sensitive to deposition conditions\(^ {18}\) leading to some inconsistency in the ellipsometric thicknesses reported in the literature\(^ {18,19,29-31}\). Furthermore, the absolute thickness values are sensitive to errors in the determination of the silicon oxide layer and the specific choice of dielectric constants. Thus, these absolute thickness values determined by ellipsometry should be interpreted with care. The ellipsometric thicknesses for pure C2 and pure C18 monolayers prepared using the protocol described above was measured to be \(7.0 \pm 0.4 \text{ Å}\) and \(17.5 \pm 0.4 \text{ Å}\), respectively (Figure 2.1a), in reasonable agreement with the values reported in the literature\(^ {19}\). Importantly, since the monolayers were all prepared and characterized under the same
conditions, these measurements allow the relative change in the monolayer thickness as a function of the mole fraction of C18 in the deposition solution ($\chi_{C18}$) to be sufficient for deriving a relationship between the C18 surface coverage ($\chi_{C18}^2$) and $\chi_{C18}$.

The mechanism of alkylsilane self-assembly on silicon has been extensively studied, and provides a basis for developing a model of long and short chain alkylsilane adsorption on silicon. Alkylsilanes assemble on silicon surfaces through a combination of self-assembly via island growth\textsuperscript{16,17}, covalent attachment via Si-O-Si bonds\textsuperscript{19}, and horizontal and vertical polymerization between silane groups\textsuperscript{19}. These modes of assembly are all characteristic of an assembly process that is reaction limited. This allows us to develop a model based on apparent surface affinity differences between C2 and C18. Because SAM growth on a solid surface is a complex process, the apparent affinity ratio is formulated as a semi-empirical constant, $n$, that potentially accounts for many differences that may arise from the possible modes of assembly discussed above.

A model was developed to obtain a relationship between $\chi_{C18}^2$ and $\chi_{C18}$ from the measured film thickness by considering that C2 has an apparent affinity for the solution/solid interface that is $n$ times greater than that of C18. The mixed monolayer film thickness ($h$) is expressed as a weighted average based on the relative concentrations of C18 and C2 at the interface and thicknesses of the pure C18 and C2 monolayers. From these model considerations $h$ is fit to the following equation:

$$h = \frac{\chi_{C18}(h_{C18} - h_{C2})}{\chi_{C18}(1 - n) + n} + h_{C2} \quad \text{(eq. 2.1)}$$
where \( h_{C18} \) is the thickness of a purely C18 monolayer and \( h_{C2} \) is the thickness of a purely C2 monolayer. Fitting the model to the film thickness measurements (see main text), using a weighted non-linear regression with \( n \), \( h_{C18} \), and \( h_{C2} \) as free parameters, yielded the model fit shown in Figure 2.1a with \( n = 1.0 \pm 0.3 \), \( h_{C18} = 18.1 \pm 0.9 \) Å, and \( h_{C2} = 6.5 \pm 0.6 \) Å. It should be noted that the film thickness of pure C2 and C18 monolayers were considered free parameters to allow for the best possible fit to the experimental data. If we were to leave these as measured values, the fit would force itself to intersect at the measured values which would imply 100% confidence in the thickness of the pure monolayers. By considering the thickness of the pure monolayers to be free parameters we can weight each data point to the experimental uncertainties, allowing for an unbiased fit. The fitted parameters demonstrated consistency with experimentally measured and reported results for the film thickness and the value of \( n = 1.0 \pm 0.3 \) obtained from the fit indicated a linear dependence of thickness (i.e. coverage) on bulk concentration. This result indicates that C2 becomes incorporated into the mixed SAM with the same apparent affinity as C18. Using the fitted parameter, \( n \), an analytical equation for determining \( \chi_{C18}^\sigma \) from \( \chi_{C18} \) was obtained:

\[
\chi_{C18}^\sigma = \frac{\chi_{C18} - \chi_{C18}}{n(1 - \chi_{C18}) + \chi_{C18}} \quad \text{(eq. 2.2)}
\]

Figure 2.1b shows this dependence of \( \chi_{C18}^\sigma \) on \( \chi_{C18} \) illustrating that C2 becomes incorporated into the mixed SAM with the same apparent affinity as C18. The error in the measured values increases with increasing \( \chi_{C18} \) due to the propagation of error completed in the calculation of \( \chi_{C18}^\sigma \).
2.3 Results and Discussion

2.3.1 Liquid Crystal Anchoring on Mixed SAMs

Since surfaces functionalized with short chains (C1,C2,C4) and C18 SAMs induce planar and homeotropic LC anchoring respectively, a similar long-chain/short-chain (i.e. DMOAP/MAP) mixed monolayer has been shown to decrease the planar anchoring strength with increasing DMOAP mole fraction, we hypothesized that an LC anchoring transition would be observed as a function of $\chi_{C18}$. This was indeed observed, as shown in Figure 2.2. Figures 2.2a and 2.2i show images consistent with the expected planar and homeotropic anchoring for C2 and C18 functionalized surfaces, respectively. Figures 2.2b-2.2h shows the transition from tilted to homeotropic LC orientation with increasing $\chi_{C18}$. The transition was found to occur between $\chi_{C18} = 0.05$ (where the anchoring was tilted) and $\chi_{C18} = 0.11$ where (weak) homeotropic orientation was observed. Furthermore, an anchoring transition occurs between $\chi_{C18}=0.024$ and $\chi_{C18}=0.049$ for a C1/C18 mixed monolayer and between $\chi_{C18}=0.00$ and $\chi_{C18}=0.029$ for a C4/C18 mixed monolayer (Figure 2.3). While systematic trends associated with chain length are not readily apparent, it is clear that the anchoring transition occurs at low $\chi_{C18}$ independent of the monolayer compositions studied.

![Figure 2.2 – LC Anchoring Transition: Polarized microscopy images demonstrating the LC orientation at varying $\chi_{C18}$. The surface coverage of C18 ($\chi_{C18}$), shown on the top of each image, was calculated from a model fit to ellipsometric measurements of the monolayer thickness (see main text). All scale bars are 100 μm.](image)
The LC anchoring transition was observed to occur at a $\chi_{C1}$ of long chain hydrocarbons well below complete coverage ($\chi_{C1}^0 \leq 0.11\pm0.04$). While the precise mechanism of homeotropic alignment on surfaces treated with a long chain SAM is not known, previous reports suggest the importance of LC infiltration into the nm-scale SAM environment. For example, second harmonic generation studies on an 8CB film supported on a DMOAP functionalized surface demonstrated that LC molecules can infiltrate the fluid DMOAP monolayer environment. Furthermore, LC alignment studies on a mixed monolayer of alkanethiols on gold demonstrated that homeotropic alignment was not induced by a purely long alkyl chain monolayer, while mixed monolayers did cause homeotropic alignment, a phenomena partially attributed to the fluidity of the top layer of a mixed monolayer. We speculate that similar behavior is observed in the mixed monolayers described here. At sufficiently high C18 coverage, the LC molecules infiltrate a fluid top layer and interact with the alkyl chains cooperatively so that the LC molecules as well as the alkyl chains align perpendicular to the interface (Figure 2.4b, 2.4c), resulting in homeotropic LC orientation. However, if the C18 surface
coverage is too low, the long alkyl chains will lose their ordered structure and the LC orientation will be non-homogeneous, resulting in tilted/planar LC orientation (Figure 2.4d). This mechanism of LC orientation driven by steric interactions can be applied to a wide range of LC molecules (e.g. non-nitrile containing LC) as it is not limited to a specific coordination interaction as is the case with homeotropic alignment on surfaces functionalized with metal salts.

2.3.2 Contact Angles on Mixed SAMs

As expected, we found that increased presence of C18 at the interface resulted in systematically higher water contact angles as shown in Figure 2.5. Consistent with previous studies involving mixed monolayers of long and short chain alkylsilanes\(^ {29,35-37}\), the contact angles of both organic liquids exhibited a non-monotonic dependence of contact angle on C18 concentration. For example, as \(\chi_{C18}\) (and therefore \(\chi^{a}_{C18}\)) decreased from unity (\(\chi_{C18}=1.0\)), the E7 contact angle (open circles in Figure 2.5) initially decreased from a maximum of 47.4\(\pm\)0.5° to a minimum of 14.4\(\pm\)0.1° at \(\chi_{C18}=0.26\). At lower concentrations of C18, the LC contact angle gradually increased with decrease
in $\chi_{C18}^\theta$ to 29.9±0.5° for a pure C2 monolayer. The isotropic oil, N26, exhibited a similar non-monotonic behavior (filled circles in Figure 2.5), suggesting that this behavior was not associated with the anisotropic physical properties of the LC. This is further supported by the observation that the composition corresponding to the contact angle minimum does not correspond to an LC anchoring transition, as observed not only for the C2/C18 mixed monolayer, but also for either a C1/C18 or C4/C18 mixed monolayer. (Figure 2.6) A C1/C18 mixed monolayer exhibited a minimum in the LC contact angle at $\chi_{C18}=0.17$ of 13.9° ± 0.4°, near perfect agreement with that observed for a C2/C18 mixed monolayer. The C4/C18 mixed monolayer exhibited a minimum in the LC contact angle at $\chi_{C18}=0.51$ of 28.6° ± 0.3°. These series of mixed monolayers comprised of either C1/C18 or C4/C18 also demonstrated the same qualitative contact angle dependence on $\chi_{C18}$ for the isotropic oil and LC suggesting that, independent of mixed monolayer composition, the anisotropic contribution to the overall surface energy is a relatively small component. Studies have suggested that the non-monotonic dependence of contact angle on coverage may be due to a varying

Figure 2.5 – Contact Angle: The contact angle of several fluids on a C2/C18 mixed monolayer at varying $\chi_{C18}$. The surface coverage of C18 ($\chi_{C18}^\theta$) shown on the top axis was calculated from a model fit to measurements of the monolayer thickness as described in the text (Error bars are omitted as they are smaller than the size of the symbols used)
coverage of surface methyl and methylene groups\textsuperscript{29,35-37}, while the monotonic decrease observed for water is due to a relatively small difference between the water contact angle on purely methyl and methylene surfaces\textsuperscript{38}.

2.3.3 Application to Micro-Structured Substrates

To explicitly demonstrate the ability of mixed monolayers to improve LC spreading and stability on micro-structured substrates, a micro-pillar array was functionalized with a C18 SAM and a mixed C2/C18 SAM ($\chi_{C18} = 0.26$). The LC wettability and the LC orientation were determined on these two substrates by measuring the LC contact angle and imaging the LC film between crossed polarizers. If the LC infiltrates the micro-pillar array, the relevant LC orientation at the SAM-LC interface can be
determined by observing the light transmission in the regions between the micro-pillars, since the LC adopts a homeotropic orientation at the opposing air-LC interface\textsuperscript{39}. However, the micro-pillar surface (which is modified by the silane deposition) induces homeotropic LC orientation relative to the micro-pillar surface (or planar relative to the specimen plane), as illustrated in Figure 2.7f. Therefore, birefringence is always observed around the edges of the micro-pillar if the LC is able to infiltrate the micro-pillar array, making the area around the micro-pillars visible as small arcs when the sample is viewed between crossed polarizers\textsuperscript{5}. However, if the LC does not infiltrate the micro-pillar array, the micro-pillars are not visible because they are surrounded by air, an isotropic medium. Thus, the presence of birefringent arcs is a signature characteristic of the presence of LC in the array.

Figure 2.7 – Sensor Wetting: LC droplets in contact with modified micro-pillar arrays. (a) Polarized light image (top-view) of a C18-functionalized array. The region above the dotted line shows the macroscopic LC droplet. (b) Side-view image of a C18-functionalized array. (c) Polarized light image (top-view) of a C18/C2-functionalized array ($\chi_{C18}=0.26$). The region above the dotted line shows the macroscopic LC droplet. (d) Side-view image of a C18/C2-modified array ($\chi_{C18}=0.26$). (e) The outlined region from part (c) enlarged 4.25X to illustrate the birefringence around the pillars and LC orientation in between the pillars. (f) A schematic illustrating the LC orientation when homeotropic orientation is induced on the SAM in between pillars (not to scale).
We found that for a micro-pillar array functionalized with a C18 monolayer, the LC did not infiltrate the array. Figure 2.7a shows a polarized microscopic image of a LC drop on the C18 functionalized array. In this image, the LC drop is visible as a semicircle at the top of the image (outlined by the area above the dashed line), demonstrating the expected birefringence due to the thickness and curvature of the LC drop, which covers the tops of the micro-pillars in this region. However, the micro-pillars outside of the LC drop are not visible, since the LC did not infiltrate the array. Furthermore, a relatively large contact angle on the micro-pillar array functionalized with pure C18 was directly observed as shown in Figure 2.7b. In this image the micro-pillar array is to the right of the drop, and a monolayer on planar glass is to the left of the drop. It is seen that the contact angle on the micro-pillar array is similar to that on the glass indicating that the interfacial energy causes the LC drop to bead on the array and prevents infiltration among the micro-pillars.

On the other hand, when the micro-pillar array was functionalized with an appropriate mixed C2/C18 SAM ($\chi_{C18}=0.26$), the interfacial surface energy promotes low LC contact angle and spontaneous infiltration of LC among the micro-pillars. High birefringence was observed in the area occupied by the actual bulk LC drop (Figure 2.7c) since the drop covers the top of the micro-pillars (again, outlined by the area above the dashed line). In contrast to the array functionalized with the C18 monolayer, however, the micro-pillars outside the bulk LC drop were visible, surrounded by small arcs (Figure 2.7c, 2.7e); less bright than in the bulk LC drop, indicating that a thin LC layer successfully infiltrated the micro-pillar array. Homeotropic orientation was maintained in the areas between the micro-pillars as evidenced by the dark appearance of the area between the micropillars, in agreement with the observation in LC cells. Further verification that the LC
infiltrated the micro-pillar array is shown in Figure 2.7d. A side view of the LC drop was captured on the mixed monolayer micro-pillar array. The interfacial energy promotes spontaneous spreading of the LC droplet with a very small contact angle that is difficult to measure, providing direct evidence for improved LC wetting in the post array.

2.4 Conclusions
Surface functionalization with a mixed monolayer comprised of long and short chain alkylsilanes provided a robust strategy for controlling surface properties that are critical to LC sensor applications. The LC contact angle was reduced from $47.4^\circ \pm 0.5^\circ$ on a purely C18 monolayer to $14.4^\circ \pm 0.1^\circ$ when the surface coverage density drops to $\chi_{C18}^d = 0.26$. Non-monotonic behavior of the LC contact angle with respect to $\chi_{C18}^d$ was observed and attributed to the isotropic contribution of the surface energy. A LC anchoring transition from planar to homeotropic LC orientation was observed for a $C2/C18$ mixed monolayer with $\chi_{C18}^d \geq 0.11 \pm 0.04$, allowing for the design of a mixed monolayer that minimized the LC contact angle while maintaining homeotropic orientation. A surface functionalized with a mixed SAM was designed that allowed for homeotropic LC orientation with good LC wetting at $\chi_{C18}^d = 0.26 \pm 0.09$ and was used to directly demonstrate the improved LC infiltration into a micro-structured substrate illustrating the practical value of this approach.

2.5 References


Chapter 3: Surfactant-DNA Interactions at the Liquid Crystal / Aqueous Interface

The presence of single-stranded (ssDNA) vs. double-stranded (dsDNA) DNA at a surfactant-laden aqueous-nematic liquid crystal (LC) interface results in distinctly different orientations of the LC molecular axis; this is of practical interest as a method to detect DNA hybridization. Results presented here provide new insights into the molecular-level mechanisms of these phenomena. The adsorption of ssDNA to a cationic surfactant-laden aqueous-LC interface caused LC reorientation, leading to coexistence between homeotropic and planar (birefringent) oriented regions. Fluorescence microscopy revealed that ssDNA preferentially partitioned into the birefringent regions, presumably causing a decreased surface coverage of surfactant and the resultant planar LC orientation. Both electrostatic and hydrophobic effects were found to be critical to inducing LC reorientation. In particular, insufficient ssDNA adsorption occurred in the absence of a cationic surfactant (e.g. with no surfactant or with a non-ionic surfactant), demonstrating the importance of electrostatic interactions with the polyanionic ssDNA. Even in the presence of a cationic surfactant, however, polyanions without hydrophobic side-group moieties (poly[acrylic acid] and dsDNA) caused no LC reorientation, while polyanions with hydrophobic side groups (polystyrene sulfonate and ssDNA) initiated the desired LC reorientation. These observations are consistent with the fact that interfacial hybridization of adsorbed probe ssDNA to complementary target ssDNA caused a reorientation from planar back to homeotropic. We propose that ssDNA forms an electrostatic interfacial complex with cationic surfactant where the hydrophobic nucleobases associate directly with the LC phase, effectively competing with surfactant molecules for interfacial sites. Upon hybridization, the hydrophobic character of the ssDNA is lost and the
nucleobases no longer associate directly with the LC phase, allowing the surfactant molecules to pack more closely at the interface.
3.1 Introduction

The detection of DNA hybridization events has applications in a wide range of disciplines including the agricultural\(^3\) and food industries\(^3\), infectious disease detection\(^3\), and gene expression\(^4\). Examples of common technologies available to detect hybridization events include RT-PCR\(^5\), colorimetric\(^6\), and fluorescence based assays\(^7\). While these strategies are well established and in some cases commercially-available\(^8,9\), the associated technologies have limitations that affect their potential for point-of-care (POC) applications, e.g. some approaches are sufficiently reliable for a lab setting but are not robust enough for POC devices. Multiplexing has been demonstrated using certain well-established strategies\(^10\); however, poorly-controlled nonspecific DNA binding to surfaces commonly used in microarray applications\(^11,12\) reduces sensitivity, creates “false positive” responses, and limits the utility of these devices in quantitative analysis. Thus, there is a need for a DNA hybridization detection strategy that eliminates these issues while maintaining the high performance of these successful approaches.

Liquid crystal (LC) – based sensors have demonstrated potential as a platform for the detection of DNA hybridization events\(^13-15\), harmful gases\(^16,17\), protein reactions\(^18\), and ssDNA quantification\(^19,20\). LC materials exhibit states of matter that are intermediate between a true crystal and an isotropic fluid. Several LC phases exist with varying degrees of order, but the one most commonly-used in sensor applications is the nematic phase of thermotropic LCs, which possesses orientational order and lacks translational order. Orientational order leads to optical birefringence that is readily detected using polarized light; this provides a convenient way to determine molecular orientation. LC sensors typically operate by detecting a perturbation in the LC orientation caused by the presence of an analyte. This strategy offers several potential advantages in microarray applications.
LC sensors generally involve a label-free detection strategy that specifically detects the presence of an analyte, thus, reducing the risk of potential false positive signals due to nonspecific binding events. Furthermore, LC materials can be obtained at a low cost, provide a read-out signal (i.e. presence or absence of optical birefringence) that can be observed by the naked eye, and can be readily developed into a robust device making this strategy attractive for POC applications. Furthermore, LCs have an intrinsic elastic energy that causes a small perturbation in the LC alignment to propagate over distances on the order of 1 to 100 μm; a natural amplification effect that provides a macroscopic response to a microscopic perturbation.\textsuperscript{14} While LC sensor applications are promising, the molecular mechanisms associated with the underlying phenomena are generally poorly understood.

Several strategies for the detection of DNA hybridization using a LC platform have been reported. In one approach, a solid substrate decorated with immobilized peptide-nucleic acids was shown to

![Image](image-url)

**Figure 3.1** – LC response to ssDNA adsorption and hybridization: Polarized microscopy images of the aqueous/LC interface laden with (a) OTAB, (b) after subsequent adsorption of ssDNA, and (c) after interfacial hybridization.
induce planar LC anchoring; sequential hybridization and surfactant adsorption at the interface resulted in a transition to homeotropic anchoring.\textsuperscript{21} Another strategy detected different LC orientations induced by either single-stranded (ssDNA) or double-stranded DNA (dsDNA) combed onto a solid substrate.\textsuperscript{14} In previous work by our research group, it was observed that the adsorption of ssDNA to a cationic surfactant-laden aqueous-LC interface induced a LC anchoring transition from homeotropic to planar (Figure 3.1a,b) and subsequent DNA hybridization caused a transition back to homeotropic LC orientation (Figure 3.1b,c).\textsuperscript{15} Our objective here was to develop a mechanistic understanding of these particular phenomena, i.e. the LC reorientation upon DNA adsorption and hybridization at an octadecyltrimethylammonium bromide (OTAB) aqueous-LC interface.

The interactions between DNA and surfactants have previously been studied in several contexts, and suggest complex effects due to both electrostatic and hydrophobic associations. Electrostatic interactions contribute to the creation of DNA-surfactant complexes in bulk solution to form aggregates at surfactant concentrations below the critical micelle concentration (cmc)\textsuperscript{22}, and dsDNA is also found to form complexes with cationic surfactants at the air-water interface\textsuperscript{23,24}. The cationic surfactants in these DNA-surfactant complexes create an environment that increases the melting temperature of dsDNA (i.e. they promote hybridization).\textsuperscript{25,26} At the air-water interface, cationic surfactants with an amine head group (ie. hydrogen-bonding moiety) have been shown to denature DNA into its single-stranded form\textsuperscript{24}, also resulting in a decreased interfacial concentration of the surfactant.\textsuperscript{24,26} With respect to hydrophobic effects, it has been suggested that exposed hydrophobic bases of ssDNA result in a further reduction of the cmc in DNA-surfactant complexes.\textsuperscript{27}
Furthermore, significant adsorption of ssDNA to uncharged hydrophobic surfaces has been demonstrated, and attributed to the hydrophobic nature of the exposed bases of ssDNA.  

These previous studies suggest that both electrostatic and hydrophobic interactions will likely play a critical role in the system studied here, while the role of hydrogen bonding would appear to be less significant since the surfactants under consideration (Figure 3.2) lack a hydrogen bonding moiety. Electrostatics is omnipresent when considering the interaction between a polyanion and a cationic surfactant; however, hydrophobic interactions, while of critical importance, will be particularly relevant to certain phenomena (e.g. ssDNA conformation). The experiments described here were designed to elucidate the relative importance of these various interactions with respect to the mechanisms associated with the LC orientational response. In particular, the LC response to polyanion adsorption was measured as a function of polyanion hydrophobicity, surfactant charge, and surfactant coverage. We also report measurements of DNA mobility and DNA partitioning to either homeotropic or birefringent domains.

3.2 Materials and Methods

3.2.1 LC Film Preparation and Polyanion Addition

LC optical cells consisted of an aqueous/LC interface, where adsorbate molecules were introduced, and an opposing solid/LC interface that induced homeotropic orientation. These cells were housed in wells that were prepared by punching a \(~5\) mm diameter hole in 2.5 mm thick silicone rubber sheets (Sigma-Aldrich) using a standard hole punch. A sheet containing one or more wells was placed onto a soda lime glass microscope slide (Corning Inc.) that had been modified with an OTES SAM (see below), and an electron microscopy grid (Electron Microscopy Sciences) was placed on
the slide in the center of each well. In order to adsorb surfactants at the aqueous/LC interface, surfactant molecules were dissolved into the LC phase. Figure 3.2 shows the different surfactants used. We used three cationic surfactants: dioctadecyldimethylammonium bromide (DODAB), octadecyltrimethylammonium bromide (OTAB), and dodecyltrimethylammonium bromide (DTAB) (Sigma). We also used the nonionic surfactant tetraethylene glycol (C12E4) (Sigma). Stock solutions of surfactant were prepared in chloroform (Fischer), known volumes of which were then added to LC (either 5CB or E7, Merck KGaA, both LC materials are thermotropic and exhibit a nematic phase at the operating conditions used; T=25°C) at the desired concentration. This mixture was then dried under a stream of ultrapure N2 (Airgas), evaporating the chloroform from the LC and leaving a mixture of surfactant dissolved in LC at a desired concentration. A micropipette was used to add 250nL of the surfactant/LC mixture to the electron microscopy grid at a temperature above the nematic-to-isotropic transition temperature of the particular LC (>35 ºC for 5CB and >60ºC for E7). Excess LC was then removed from the grid via capillary action through contact with a clean micro capillary tube. The LC film was then allowed to slowly cool to room temperature and the LC cell was placed onto the microscope stage. The silicone well was then filled (~50μL) with either an aqueous solution of 5mM NaCl (pH ~5.5-6) for most experiments or a 10 mM Tris-Cl, 1mM EDTA buffer (pH ~7.5) for pure dsDNA addition (see below) and was then imaged between crossed polarizers using a Nikon Eclipse Ti microscope equipped with a Nikon DS-Fi1 color C-MOS camera.
Polyanions were added through the aqueous phase in order to measure their effect on the LC orientation upon adsorption, the mobility of certain polyanions at the interface (see below), and the partitioning of DNA between LC domains. Prior to the addition of polyanion, the LC orientation due to the adsorption of surfactant was imaged between crossed polarizers. Unlabeled ssDNA (probe) (5’AGAAAAACTTCGTGC3’; Biosearch), fluorescently labeled ssDNA probe (3’-modified with AlexaFluor 568; Biosearch), purified dsDNA (see below), polystyrene sulfonate (PSS; Figure 3.2) (75,000 MW, Sigma), or poly (acrylic acid) (PAA; Figure 3.2) (1,800 MW, Sigma) was added to the aqueous phase at concentrations of 2.5 μM, 2.5 μM, 2.5 μM, 14 μM, and 14 μM respectively. The LC orientation was continuously monitored using polarized microscopy. When applicable, the DNA
partitioning was also monitored with epifluorescence microscopy (Nikon Eclipse Ti microscope outfitted with a second camera; Photometrics Cascade 512B). In experiments that involved studying the LC response to interfacial hybridization, a solution containing complementary ssDNA (target) was subsequently added to the aqueous phase. If fluorescent probes were used in the first polyanion addition a buffer exchange was completed prior to target addition, to remove any background fluorescence due to unabsorbed fluorescent probes. Either unlabeled target (5’GCACGAAGTTTTTCT3’, Biosearch) or fluorescently labeled target (3’-modified with fluorescein, Biosearch) was added to the aqueous phase (~1–10 pmol) and the LC orientation was continuously monitored using polarized microscopy; again the DNA partitioning was monitored using epifluorescence microscopy when applicable.

3.2.2 OTES Preparation

Self-assembled monolayers (SAMs) of octadecyltriethoxysilane (OTES) (Gelest Inc.) were prepared according to published procedures. Soda lime glass microscope slides (Corning Inc.) were cleaned sequentially with 2% aqueous micro-90, deionized water (18.2 MΩ), and piranha solution (30% aqueous H₂O₂ (Fisher Scientific) and concentrated H₂SO₄ (Fisher Scientific) 1:3, v/v) at 80°C for 1 hr. (Warning: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store in closed container). After piranha cleaning, the microscope slides were rinsed with deionized water (18.2 MΩ) and dried under a stream of ultrapure N₂. A deposition solution of n-butylamine (Fisher Scientific) and OTES was prepared in toluene (Fisher Scientific) at 1:3:200 volumetric ratios, respectively. The deposition solution was warmed to 60°C, clean and dry microscope slides were first rinsed with toluene, and then submerged in the warm deposition solution. The slides were incubated in the deposition solution for 1 hour at 60°C. Upon removal,
the slides were rinsed with toluene, dried under a stream of ultrapure N₂, and stored at room temperature in a vacuum desiccator. A custom-built contact angle goniometer was used to verify that the water contact angle (θₛ, measured via the static sessile drop method) of the prepared SAMs was sufficient to achieve strong homeotropic anchoring (θₛ > 95°).

3.2.3 dsDNA Preparation

Two reverse complementary ssDNA strands (5'TATTAGGGATGAAGGGCACGAAGTTTTTTCT3'; 5'AGAAAAACTTCGCTCCCTCATCCCCTAATA3'; Integrated DNA Technologies) were annealed by adding equal parts into a microcentrifuge tube, heating to 95 °C for 5 min and then slowly cooling to room temperature for 2 hours. The annealed dsDNA (Tₘ = 61.1 °C) was then treated with exonuclease I (New England Biolabs), suspended in 67 mM Glycine-KOH, 6.7 mM MgCl₂, and 10 mM 2-Mercatoethanol at pH~9.5 (New England Biolabs) for 1 hour to digest any residual ssDNA in solution into individual nucleotides. The dsDNA solution was then purified with a QIAquick nucleotide removal kit (Qiagen) to separate the residual nucleotides and enzyme from the annealed dsDNA. The purified dsDNA pellet was resuspended in 10 mM Tris-Cl and 1 mM EDTA buffer (Qiagen). An aliquot of purified dsDNA was stained with ethidium bromide, xylene cyanol, and bromophenol blue then electrophoresis was run in a 15% acrylamide gel for 1hr with buffer conditions of 89 mM Tris-Cl, 89 mM Borate, 2mM EDTA buffer (Sigma) to verify that the DNA was in fact dsDNA. Furthermore, UV-vis analysis indicated that the purified dsDNA was free of any impurities. The remaining purified dsDNA was then precipitated with ethanol and sodium acetate and resuspended to 39 μM.
3.2.4 Flow Cell Assembly

In certain experiments a LC film was prepared in a flow cell environment to facilitate an efficient buffer exchange of the aqueous phase. Mixtures of surfactant in LC were prepared as described above. An electron microscopy grid was placed onto a 1” diameter circular glass cover slip (Electron Microscopy Sciences). The desired mixture of surfactant in LC was then housed within the pores of the electron microscopy grid as described in the main text and the entire glass cover slip was placed onto the base of the flow cell assembly. The base of the flow cell assembly contains a circular opening, slightly less than 1” in diameter, in which an o-ring (Kalrez, McMaster-Carr) of the proper diameter was placed. The cover slip was then placed on top of this o-ring. Custom fabricated Teflon spacers (~240μM thick) designed with a hole in the center was placed directly on top of the glass cover slip to create a gap between the cover slip and the top glass (fused silica, Mark optics). The base of the flow cell has channels built into it that allow for one to introduce flow through this gap created by the Teflon spacers. The top glass slide was fastened into place by the top piece of the flow cell assembly through which screws were used to clamp the entire flow cell assembly together. Buffer was introduced through the inlet port using care to flush out any air bubbles that may be present in the cavity created by the Teflon spacers. Once assembly is complete, ssDNA was introduced through the inlet port at the desired concentration and at a total load of ~250μL to ensure that the entire volume in the flow cell cavity was flushed out. When a buffer exchange of the aqueous phase was conducted, excess volumes on the order of ~0.5-1mL of the buffer were introduced through the inlet port to ensure an efficient buffer exchange.
3.2.5 Fluorescence Recovery after Photobleaching (FRAP)

LC films were prepared as described above with varying bulk concentrations of OTAB in LC (40, 80, 100, 140, or 200 μM) and contained within a flow cell environment instead of the micro well environment previously described. An aqueous solution of 5mM NaCl (pH=5.5-6) was introduced to the flow cell and incubated for 5-10 min. A 2.5μM solution of fluorescently labeled ssDNA (5’AGAAAAACTTCGTGC3’ – Fluorescein; Invitrogen) was then introduced and incubated for an additional 5-10 min. A buffer exchange of the bulk aqueous phase was completed by flowing ~1mL of aqueous 5mM NaCl (pH=5.5-6) through the flow cell to remove background fluorescence due to the presence of the fluorescent ssDNA. The LC orientation was measured using polarized light microscopy to evaluate homeotropic or planar LC orientation. To photobleach a small circular region at the interface, the aperture on the microscope was closed to its smallest size and the sample was illuminated for ~10 sec using a 120W Mercury Vapor Short Arc lamp (model CHGFIE, Nikon). The fluorescence recovery was then measured by opening the aperture and collecting a time series of images for 10-40 minutes. The fluorescence intensity was analyzed using ImageJ (NIH Freeware) and the fractional fluorescence recovery curve \( f_K(t) \) was calculated:

\[
f_K(t) = \frac{F_K(t) - F_K(0)}{F_K(\infty) - F_K(0)} \quad \text{(eq. 3.1)}
\]

where \( F_K(t) \) is the average intensity of the photobleached area at time \( t \), and \( F_K(\infty) \) is the average intensity of the region surrounding the photobleached area. A custom written Mathematica code was used to fit the fractional recovery function to the equation \(^{29}\):

\[
f_K(t) = Ae^{-2\tau/t}[I_0(2\tau/t) + I_1(2\tau/t)] \quad \text{(eq. 3.2)}
\]

where \( \tau \) is the time constant used as the free parameter, \( A \) is a constant which represents the fraction of mobile molecules (constrained to \( A=1 \)), \( t \) is time, \( I_0 \) is a zero-order modified Bessel
function of the first kind and $I_1$ is a first order modified Bessel function of the first kind. The time constant, $\tau$, was then used to calculate the diffusion coefficient ($D$):

$$D = \frac{r^2}{4\tau}$$

where $r$ is the radius of the circular photobleached area.

3.3 Results

3.3.1 ssDNA Adsorption and LC Reorientation

In previous work, we found that under appropriate conditions, adsorption of ssDNA to an OTAB laden aqueous-LC interface induced a reorientation of the LC director from homeotropic to planar. As shown in Figure 3.3, systematic studies found that this reorientation occurred only when operating at an OTAB surface coverage near the minimum required for homeotropic orientation, which corresponded to a bulk [OTAB] $\approx$ 100 µM in our experimental system. At a bulk [OTAB] well below 100 µM (Figure 3.3a–3.3c), the LC orientation was unaffected by the adsorption of ssDNA (i.e. the LC remained birefringent), while at a bulk [OTAB] sufficiently greater than 100 µM (Figure 3.3g-3.3i), homeotropic LC orientation was observed regardless of the presence of ssDNA at the interface (although a transient nucleation of small birefringent domains was sometimes seen; Figure 3.3h). At an [OTAB] $\approx$100 µM, where the surface coverage was near the minimum required for homeotropic orientation (Figure 3.3d-3.3f) a LC reorientation was observed upon ssDNA adsorption. In particular, small birefringent domains nucleated (Figure 3.3e) and eventually coalesced to form large domains of stable planar LC orientation (Figure 3.3f).
3.3.2 Cationic and Nonionic Surfactant Monolayers

The LC response to ssDNA adsorption and hybridization was studied using surfactants with varying surface activity and head group charge. Three cationic surfactants with decreasing surface activity were employed (DODAB, OTAB, DTAB, respectively) as well as a nonionic surfactant (C12E4). In all cases, a homeotropic LC orientation was induced by the presence of sufficient surfactant. The same qualitative LC response was observed with all three cationic surfactants upon addition of probe ssDNA; i.e. a LC reorientation was observed from homeotropic to planar upon ssDNA adsorption (see Figure 3.2a – 3.2b). Furthermore, the characteristic response to hybridization upon addition of

Figure 3.3 – LC reorientation upon ssDNA adsorption at varying [OTAB] – Polarized light microscopy images of the LC-aqueous interface at (a-c) low [OTAB] (d-f) intermediate [OTAB] and (g-i) high [OTAB] (a,d,g) before and (b,c,e,f,h,i) after ssDNA adsorption at (b,e,h) 1min and (c,f,i) 15min.
complementary target ssDNA (see Figure 3.2b - 3.2c) was also observed for all three cationic surfactants. However, with nonionic surfactant no LC reorientation was observed upon ssDNA adsorption; in fact, little to no fluorescently-labeled ssDNA remained adsorbed to the interface following an exchange of the bulk aqueous phase. The same lack of ssDNA adsorption was observed in the absence of surfactant. These results suggest that electrostatic interactions are critical to achieve sufficient ssDNA adsorption to realize a LC response, and that the surfactant surface activity has little to no affect on the ability to achieve a LC reorientation upon interfacial hybridization.

3.3.3 ssDNA Surface Coverage and Diffusion

Fluorescence microscopy was used to measure relative surface coverage and mobility of ssDNA at the aqueous-LC interface as a function of OTAB surface coverage. At [OTAB]=100μM, the coexistence of homeotropic and birefringent domains observed following ssDNA adsorption provided an opportunity to measure the ssDNA coverage and mobility simultaneously in both planar and homeotropic regions. The diffusion coefficients obtained were on the order of 0.1-1μm²/sec as shown in Figure 3.4.
According to the Einstein relation, the diffusion coefficient, $D$, is related to the mobility, $\mu$, through the expression $D = \mu k_B T$, where $k_B$ is the Boltzmann constant and $T$ is the absolute temperature. Depending on the experimental conditions, the interfacial mobility, $\mu$, may be dominated by viscous drag from the adjacent bulk phases (the small Boussinesq number limit) or from drag associated with molecular crowding within the interfacial layer (the large Boussinesq number limit), which considers surface crowding (i.e. surface viscosity) to be the dominant determinant of surface diffusion. In the former case, the minimum diffusion coefficient one could observe is $2.78 \, \mu m^2/s$, assuming an extreme radius of gyration, $R_H = 47.2 \, \AA$, for ssDNA lying flat at the interface. This suggests that the drag on ssDNA during interfacial diffusion is dominated by surface viscosity (i.e. lateral interactions with molecules within the interfacial layer) rather than drag from the LC and aqueous phases; the measured interfacial diffusion coefficients are reasonable for this regime. For

Figure 3.4 – ssDNA Diffusion: The diffusion coefficient, measured via FRAP, of ssDNA at an OTAB laden aqueous-LC interface with varying OTAB coverage.
example a value of $D=0.6 \, \mu m^2/s$ is consistent with a molecule with $R_H=10$ Å diffusing within a layer with a surface viscosity of $1.79 \times 10^{-9}$ P-m. Therefore, the interfacial mobility provides information about the magnitude of interfacial crowding.

Notably, the diffusion coefficient was consistently smaller in regions of planar LC orientation than in regions of homeotropic LC orientation. As discussed above, smaller diffusion coefficients are indicative of increased surface crowding. Fluorescence images illustrating the partitioning of ssDNA at the interface validate this claim. Figure 3.5a shows a polarized light microscopy image of a cationic surfactant laden aqueous-LC interface after ssDNA adsorption, while Figure 3.5b shows its corresponding epifluorescence image. These images illustrate that, in general, the ssDNA surface concentration is higher within the regions of planar LC orientation than in the regions with homeotropic LC orientation, consistent with the slower diffusion in these regions. We note that some anomalous weakly-birefringent sub-domains in Figure 3.5 exhibit a lower relative ssDNA coverage. Following these anomalous regions with time, we found that they eventually transitioned to a homeotropic orientation.
3.3.4 PSS, PAA, and dsDNA Adsorption

We studied the LC response upon adsorption of PSS and PAA to isolate the role of electrostatic and hydrophobic interactions. The rationale for these experiments involved the notion that the structure of PSS is analogous to that of ssDNA in that it is polyanionic with hydrophobic sidegroup moieties, while PAA is purely polyanionic and lacks hydrophobic moieties. Excess PSS and PAA were added to the aqueous phase in contact with LC films containing [OTAB] ≈ 100 µM. In the case of PSS, we observed reorientation of the LC director from homeotropic to planar/tilted, consistent with the LC reorientation observed upon ssDNA adsorption. Interestingly, upon the addition of PAA to the OTAB-laden interface there was a negligible effect on LC alignment (i.e. the LC orientation remained homeotropic). Similarly, the addition of purified dsDNA also did not induce a LC reorientation. These results suggest that a polyanion lacking hydrophobic moieties does not have the ability to induce a change in the LC orientation at a cationic surfactant laden aqueous/LC interface.

3.3.5 Interfacial Hybridization

As observed previously\textsuperscript{15}, upon hybridization, the fluorescently labeled target DNA was concentrated in regions of homeotropic LC orientation (Figure 3.6a – 3.6b), proving that the dsDNA is preferentially found within homeotropic regions. Interestingly, we also found that when unlabeled target was hybridized to previously-adsorbed fluorescently-labeled probe (Figures 3.6c - 3.6d), the fluorescently labeled probe was also found at higher concentrations in the homeotropic regions. Combined, these two observations suggest that the interfacial packing density of dsDNA (in homeotropic regions) after hybridization was higher than the packing density of ssDNA (in birefringent regions) before hybridization.
3.4 Discussion

The results reported above suggest that attractive electrostatic interactions between the interfacially-bound surfactant and the polyanion introduced into the aqueous phase are necessary for significant polyanion adsorption to occur, but not sufficient to induce an LC reorientation from homeotropic to planar. In particular, polyanions with hydrophobic side-chains (ssDNA and PSS) were able to induce LC reorientation from homeotropic to planar following adsorption at appropriated surfactant concentrations, while polyanions without hydrophobic moieties (dsDNA and PAA) did not affect the LC orientation. This suggests that, in conjunction with electrostatic interactions between polyanions and a cationic interface, hydrophobic interactions between hydrophobic side groups and the LC material are necessary to induce a LC reorientation.

Figure 3.6 – dsDNA hybridization at an aqueous-LC interface: (a,c) Polarized light microscopy and (b,d) epifluorescence microscopy images of an OTAB laden aqueous-LC interface after hybridization using either fluorescently labeled (a,b) target or (c,d) probe.
Our results also suggest that a critical surface coverage of long-chain surfactant is necessary to induce homeotropic anchoring. Similar conclusions have been drawn from a number of previous studies where planar anchoring was observed at the LC-aqueous interface in the presence of low surfactant concentrations, and a transition to homeotropic anchoring was observed at a critical surfactant concentration.\textsuperscript{15,32,33} This is also consistent with LC anchoring on solid surfaces, where a low concentration of long alkyl chains was shown to induce planar anchoring, but a sufficiently high surface concentration of long chains caused homeotropic anchoring.\textsuperscript{34} Electrostatic effects have also been shown to affect the orientation of polar liquid crystals (like the cyanobiphenyls used here); however, the expected orientational effects due to electrostatics would be exactly opposite to what we observe. For example, the adsorption of anionic ssDNA to the cationic surface layer has the potential to produce a polar double-layer; however, it has been shown that this sort of double-layer causes homeotropic anchoring\textsuperscript{35,36}, while we observe the exact opposite trend. Therefore, it is reasonable to conclude that the orientational transitions observed upon surfactant adsorption, probe ssDNA adsorption, and target DNA hybridization are dominated by “steric” LC anchoring effects associated with changes in the local interfacial concentration of the long-chain surfactant.

We hypothesize that a reduction in the interfacial surfactant concentration can be caused by the intercalation of hydrophobic side chains (e.g. nucleobases) upon ssDNA adsorption. At sufficiently low OTAB coverage the DNA molecules are capable of intercalating between the surfactant molecules, but at higher surfactant coverage the surfactant molecules are too densely-packed for this to occur. In particular, at a [OTAB]=100μM (the minimum concentration required for homeotropic anchoring), a lower limit for the surfactant molecular area (assuming all surfactant molecules adsorb at the interface) is calculated to be 0.92 nm$^2$/molecule. This is ~5 times the close-
packed molecular area (~0.18 nm²), suggesting that a substantial amount of open space is available at the interface, roughly 0.74 nm² per surfactant molecule. The notion that such a relatively low surface concentration of long chains is able to induce hometropic anchoring is consistent with recent results at a solid surface where a long-chain surface coverage of only ~11% caused homeotropic anchoring. Given an approximate cross-sectional area of ~0.4 nm²/nucleotide in ssDNA, there is space for 1–2 nucleotides per surfactant molecule to reside within the interfacial layer and interact strongly with the LC subphase. Furthermore, ssDNA is highly flexible (persistence length ~5.9Å) allowing for the DNA molecule to bend in a way that allows these hydrophobic interactions to occur. We hypothesize that at high bulk OTAB concentrations, the interface is sufficiently crowded with surfactant molecules, that individual nucleotides can no longer easily intercalate into the layer and associate with the LC phase. In this case, the ssDNA may still bind electrostatically to the surface, but no LC reorientation is observed.

These hypotheses are supported by our observations of ssDNA interfacial diffusion as a function of OTAB concentration and LC orientation. Specifically, when the ssDNA intercalates between the surfactant molecules one would expect increased surface crowding and a commensurate decrease in diffusion. This is in agreement with our observations, where we observe higher ssDNA concentrations and slower diffusion in birefringent regions (corresponding to low OTAB concentration) and lower ssDNA concentrations and faster diffusion in homeotropic regions (corresponding to high OTAB concentration).

The results of experiments using ssDNA analogs provide further support for the idea that hydrophobic side-chain moieties intercalate between surfactant molecules to cause LC
reorientation. In particular, the hydrophobic aromatic moieties on PSS are capable of interacting with the LC phase via hydrophobic interactions in analogy with the nucleobase/LC interactions described above. In the absence of hydrophobic moieties, adsorption of a polyanion (PAA) caused no LC response. In related work, Kinsinger et al.\(^{38}\) found that the addition of a PSS salt, sodium polystyrene (SPS), to an amphiphilic polymer adsorbed to the LC/aqueous interface induced a LC reorientation from homeotropic to planar, providing further evidence that the addition of a hydrophobic polyanion to an adsorbed cationic surfactant, either free or polymeric may induce a LC reorientation. The prominent role of hydrophobic interactions as evidenced from our experimental observations and the literature further supports the theory that the LC realignment is related to the intercalation of the exposed hydrophobic bases of ssDNA intercalating between surfactant molecules and altering the coverage of surfactant.

We propose that the strongly-associated interfacial DNA-surfactant complex with intercalated nucleobases results in ssDNA configurations that are predominantly two-dimensional within the plane of the interface. While this two-dimensional confinement involves an entropic penalty, this is offset by the favorable interactions between the nucleobases and the hydrophobic LC phase. A reasonable estimate for the strength of this hydrophobic interaction is 30 cal/mol/Å\(^2\).\(^{39}\) If we assume conservatively that half of the nucleotides take part in these interactions, the total binding energy is \(~11\) kcal/mol. The approximate loss in entropy due to two-dimensional confinement of the polymer strand can be calculated as \(\Delta S = k_B \left( \ln(W_{3D}) - \ln(W_{2D}) \right)\), where \(W_{3D}\) and \(W_{2D}\) are the multiplicities of a random walk in three and two dimensions respectively. This calculation yields the value \(T\Delta S \approx 5\) kcal/mol at room temperature, suggesting that the favorable hydrophobic interactions may plausibly compensate for the loss of configurational entropy resulting from 2D confinement.
As discussed below, this hypothetical 2D confinement results in an effective entropic repulsion between surfactant molecules associated with the ssDNA chains.

### 3.4.1 Proposed Mechanism for the Adsorption and Hybridization Induced LC Reorientation

Figure 3.7 illustrates a schematic representation of the proposed mechanism. ssDNA in bulk solution (Figure 3.7a) adopts a relatively globular configuration with the hydrophobic bases partially shielded within the molecular core. The negatively-charged ssDNA is attracted to the cationic surfactant at the aqueous/LC interface via electrostatic interactions. As the ssDNA adsorbs to the interface (Figure 3.7b), short range hydrophobic interactions influence the DNA to uncoil, allowing the hydrophobic bases to intercalate between the surfactant molecules and interact with the hydrophobic LC subphase. The combination of electrostatic and hydrophobic interactions induces a two-dimensional confinement of ssDNA resulting in the increased molecular area of the cationic surfactant proposed above. The radius of gyration of a polymer scales as $N^\nu$, where $N$ is degree of...
polymerization and ν is a scaling exponent with values of ν_{3D}=0.6 in 3D and ν_{2D}=0.75 in 2D for self-avoiding walks.\textsuperscript{40} Therefore, a polymer strand is more compact in bulk solution than when confined to an interface. A ssDNA molecule will initially adsorb to the interface in its more compact form, but as it forms a complex with cationic surfactants it will tend to spread, increasing its interfacial area by a factor of ~1.5. Due to the strong association of the ssDNA with the surfactants, this increase in molecular area of the ssDNA translates to a similar increase in the molecular area of the surfactant. Therefore, when the surfactant concentration is only slightly higher than the critical concentration required for homeotropic orientation, this increase in surfactant molecular area serves to decrease the surface coverage below that required for homeotropic orientation, resulting in the observed anchoring transition.

Upon interfacial DNA hybridization (Figure 3.7c), several phenomena occur that may contribute to the observed LC reorientation. The persistence length of the DNA increases by a factor of ~140,\textsuperscript{37,41} the hydrophobic bases of the DNA are no longer exposed, and the linear charge density of the DNA approximately doubles. Each nucleobase of ssDNA prefers to bind to its complementary base than to remain intercalated between the surfactant molecules, since hybridization involves not only hydrophobic interactions among the base pairs but also hydrogen bonding and aromatic stacking. Therefore, the ssDNA strands reorganize at the interface to hybridize with their complement; and once hybridized can be considered as rigid rods with twice the linear charge density. This rigidity of the dsDNA strand allows for more efficient packing of dsDNA at the interface and its increased charge density may promote an increase in the local surface coverage of cationic surfactants. Furthermore the hydrophobic bases that were exposed on the ssDNA strand are now hidden within the core of the dsDNA helix, preventing intercalation of the bases between the surfactant molecules.
via hydrophobic intercalations. The combination of these phenomena allows for a reorganization of the surfactants at the interface to the original surface coverage prior to ssDNA adsorption (or greater), resulting in the observed anchoring transition from planar to homeotropic LC orientation.

3.5 Conclusions

Electrostatic and hydrophobic interactions were both found to be of critical importance to promote the formation of a strongly associated ssDNA-surfactant complex. This ssDNA-surfactant complex is likely confined to two dimensions resulting in an increase in the surfactant area per molecule. The increased surfactant molecular area induces a LC anchoring transition from homeotropic to planar, as the LC orientation is strongly affected by the surfactant coverage. A subsequent LC anchoring transition from planar to homeotropic upon interfacial hybridization is explained by a reorganization of the surfactant at the interface due to the dramatic changes in the DNA structure associated with hybridization. This proposed mechanism for ssDNA adsorption and hybridization not only provides insight into the driving forces behind the LC sensor described here but also has implications to other disciplines involving polymer – surfactant interactions.

3.6 References


(31) Saffman, P. G.; Delbruck, M. Proceedings of the National Academy of Sciences of the United States of America 1975, 72, 3111-3113.


Aptamer-ligand binding events, involving small molecule targets, at a surfactant-laden aqueous/liquid crystal (LC) interface were found to trigger a LC reorientation that can be observed in real-time using polarized light. The response was both sensitive and selective: reorientation was observed at target concentrations on the order of the aptamer dissociation constant, but no response was observed in control experiments with target analogues. Circular dichroism and resonance energy transfer experiments suggested that the LC reorientation was due to a conformational change of the aptamer upon target binding. Specifically, under conditions where aptamer-ligand binding induced a conformational change from a relaxed random coil to more intricate secondary structures (e.g., double helix, G-quadruplex), a transition from planar to homeotropic LC orientation was observed. These observations suggest the potential for a label-free LC-based detection system that can simultaneously respond to the presence of both small molecules and nucleic acids.
4.1 Introduction

The extraordinary physical properties of liquid crystal (LC) materials – long-range orientational order, responsiveness to external stimuli, and optical anisotropy – have made them uniquely valuable in display and optoelectronic applications. Recently, there is increasing momentum aimed at exploiting these same properties for other applications. Of particular interest are applications where the LC responds to the presence of specific molecular compounds (e.g. for environmental monitoring or molecular diagnostic applications), translating these chemical signals into simple visual cues. Here, we demonstrate a dynamic LC response to specific binding events associated with what are arguably the ultimate molecular recognition elements – aptamers – nucleic acid constructs that can be engineered to recognize a diverse range of targets. We define the mechanistic molecular principles underlying this response, specifically that the LC is influenced by the conformational change of the aptamer’s secondary structure that occurs upon target binding.

Compared to standard methods based on monoclonal antibodies, the development of new aptamers (using Systematic Evolution of Ligands by Exponential Enrichment [SELEX]) is faster, simpler, more robust, and yields aptamers that can bind selectively and with excellent sensitivity to a wide variety of targets, including small organic molecules, proteins, antibodies, and even cells. Coupled with an appropriate transduction method, aptamers could be the basis of a universal multiplexed detection strategy capable of the simultaneous detection of many different classes of analytes in the same sample.

The advantageous properties of aptamers as a molecular recognition element have inspired the development of biosensors capable of detecting aptamer-ligand binding events. Significant
progress has been made in the development of colorometric\textsuperscript{12-15}, electrochemical\textsuperscript{16-18}, fluorescence\textsuperscript{12,19,20}, and mass-sensitive\textsuperscript{21-23} strategies; however, the transduction strategies employed in these applications are fundamentally limited for multiplexed applications. When aptamer-ligand binding occurs in the bulk phase (e.g. nanoparticle colorometric and label-free fluorescence assays) a characteristic detection signal for each target species is required (e.g. fluorescence emission wavelength), placing a finite constraint on multiplexing capacity. Other strategies, such as mass-sensitive and electrochemical detection, confine the aptamer-ligand binding to an interface and thus have the potential for site-dependent multiplexing. However, signal transduction in many of these approaches is highly non-specific (e.g. surface adsorption or localization of redox species) and the presence of even small amounts of interfering species will produce a false response. To achieve a universally multiplexed aptasensor the transduction element should ideally be label-free and respond specifically to aptamer-ligand binding.

LC-based sensing schemes have proven capable of specific signal transduction through LC reorientations driven by interfacial enzymatic reactions\textsuperscript{24} or molecular binding\textsuperscript{25} events. The unique interfacial phenomena that lead to LC reorientation in these systems are complex and subtle, often involving the competition between multiple non-covalent intermolecular interactions\textsuperscript{26}. Understanding the underlying mechanisms behind these phenomena is of significant fundamental interest. Furthermore, the intrinsic cooperative behavior associated with the long-range orientational order of the LC phase provides a natural amplification effect, eliminating the need for (bio)chemical amplification, labeling, and/or expensive instrumentation. LC-based detection of aptamer binding would provide a potential path forward for label-free multiplexed un-amplified detection of multiple target types (e.g. small molecules, nucleic acids, and proteins).
simultaneous detection of multiple molecular species in a label-free sensor scheme is an important goal, with widespread applications in areas including environmental monitoring, bio/chemical warfare detection, and medical diagnostics. Here, we demonstrate the underlying principles that will enable these applications.

In previous work, we developed a system where DNA hybridization at a surfactant-laden aqueous/LC interface caused a dynamic LC reorientation. This approach exploited a LC anchoring transition from planar to homeotropic orientation caused by increasing surface concentrations of long-chain surfactants. Upon adsorption of ssDNA to a surfactant-laden aqueous/LC interface (with homeotropic LC anchoring), the proximity of hydrophobic nucleobases to the hydrophobic LC perturbs the interfacial structure of the surfactant monolayer, causing LC reorientation to a planar configuration. Upon subsequent DNA hybridization, however, the DNA became less hydrophobic, allowing for a return to the interfacial structure associated with homeotropic anchoring. Thus, the orientation of the LC phase responded directly to a change in DNA conformation; in particular, a reduction in the amount of exposed hydrophobic nucleobases.

We hypothesized that a related approach could be used to detect other binding events associated with a nucleic acid conformational change at the aqueous/LC interface. Specifically, we utilized aptamers that are known to bind to the small molecule targets adenosine and arginine. We chose these aptamers as representative examples because they represent a DNA (adenosine) and RNA (arginine) aptamer, their target molecules possess significantly different structures, and they demonstrate relatively predictable conformational changes upon binding to their target. For instance, structural studies suggest that in the absence of target, both aptamers have a
significant portion of their nucleobases exposed, a conformation required for a LC orientational transition upon adsorption at the aqueous/LC interface. As the adenosine aptamer binds to its target, it folds into a conformation with Watson-Crick base pairing at its tails and a G-quadruplex structure at its head.\textsuperscript{31} Similarly, the arginine aptamer is known to contain nucleobases within the core of the aptamer-ligand complex upon binding.\textsuperscript{32} In both cases, ligand binding induces a significant decrease in the amount of exposed nucleobases, a conformational change analogous to that of DNA hybridization. Our aims in the current study were (i) to test the hypothesis that aptamer-ligand binding at an aqueous/LC interface can result in a LC reorientation and (ii) to verify and examine the relationship between the nucleic acid conformational changes that occur and an ability to induce a LC reorientation.

Figure 4.1 – (a) Chemical structure of the aptamer targets (b) polarized light microscopy images of the aqueous/LC interface (i) laden with OTAB, (ii) after adsorption of the adenosine aptamer (2.5µM), (iii) ≈20 sec after addition of adenosine (≈300 µM), and (iv) ≈5min after addition of adenosine (c) Dynamic LC response upon addition of ligands; $f_H$: Fractional increase in homeotropic area (d) $f_H$ upon subsequent additions of either adenosine or arginine
4.2 Results and Discussion

Under the appropriate aqueous and interfacial conditions we have, in fact, demonstrated the capability for aptamer-ligand binding events to induce a LC reorientation. When a sufficiently high surface concentration of cationic octadecyltrimethylammoniumbromide (OTAB) surfactant was adsorbed at an aqueous/LC interface\textsuperscript{27,28}, the LC orientation was homeotropic as expected (Figure 4.1b(i), 4.2a). Upon adsorption of aptamer (either the adenosine- or arginine-specific aptamer) to the OTAB laden aqueous/LC interface, a transition to tilted/planar LC orientation (Figure 4.1b(ii), 4.2b) occurred, consistent with previous reports of the LC reorientation upon adsorption of unstructured ssDNA under similar conditions. This reorientation suggests that, under these aqueous conditions, the interfacial structures of both aptamers exhibited substantial ssDNA character (i.e. with exposed hydrophobic nucleobases). When the appropriate target was subsequently added, the reverse LC reorientation occurred, characterized by the nucleation and growth of small homeotropic domains (Figure 4.1b(iii), 4.2c) that eventually coalesced to give a consistent homeotropic orientation (Figure 4.1b(iv), 4.2d). This LC response mimicked the LC

Figure 4.2 – Polarized light microscopy images of the aqueous/LC interface (a) laden with OTAB, (b) after adsorption of the arginine aptamer (2.5µM), (c) ≈20sec after addition of arginine (≈1mM), and (d) ≈5min after addition of arginine
response upon DNA hybridization under the same interfacial conditions, suggesting that the conformational changes that occurred were also analogous.

Several control experiments were performed in order to demonstrate the specificity of the LC response to aptamer binding. The LC response upon addition of cytidine, thymidine, and guanosine 5′ monophosphate (GMP) to an adenosine aptamer laden interface, and upon addition of citrulline to an arginine aptamer laden interface, was tested. Furthermore, the LC response upon addition of adenosine to an interface laden with an adenosine aptamer containing a single base mismatch\(^{31}\) was also tested. In all of these control experiments, no LC reorientation was observed (e.g. Figure 4.3) under the appropriate aqueous conditions (2.5mM Na\(_2\)PO\(_4\), pH=7.3; methods). To further
illustrate this point, we measured the time-dependence of the increase in fractional homeotropic area ($f_H$) extracted from polarized light microscopy images, providing a quantitative signature of the LC reorientation (Figure 4.1c, Figure 4.4). In these experiments, the addition of the appropriate aptamer consistently induced a transition to tilted/planar orientation. When adenosine or arginine was added ~30 sec after stabilization of the planar LC orientation ($t_i$) a distinctive increase in homeotropic coverage was observed over the following several minutes (solid black curves in Figure 4.1c and 4.4). If GMP or citrulline was instead added at $t_i$, no increase in the homeotropic coverage was observed (dotted red curves in Figure 4.1c and 4.4) until adenosine or arginine was subsequently added. These experiments demonstrated specificity consistent with the requirements for multiplexed detection, as a target-specific LC reorientation occurred in the presence of interfering ligands with very similar structures to the target.

The sensitivity and quantitative nature of the LC response was tested by “dose-response” experiments (Figure 4.1d). Small amounts of adenosine or arginine were added to the aqueous
phase after adsorption of the appropriate aptamer at an OTAB laden aqueous/LC interface; polarized light microscopy images were obtained upon stabilization of the LC orientation. A LC reorientation, characteristic of the specific response described above, was observed at bulk concentrations consistent with previously reported dissociation constants for aptamer-ligand binding\textsuperscript{7,30} (dashed lines in Figure 4.1d). This systematic increase in homeotropic coverage observed with increasing concentrations of ligand provided a direct correlation between the LC reorientation and aptamer-ligand complex formation.

The operating conditions used in the experiments described above involved a relatively low ionic strength ([Na\textsubscript{2}PO\textsubscript{4}H] ≈ 2.5mM). Under these conditions we consistently observed a significant increase in the planar LC area upon adsorption of either aptamer (Figures 4.5a, 4.5c; Table 4.1) and a subsequent increase in the homeotropic area upon aptamer-ligand binding (Figures 4.5b, 4.5d; Table 4.1). However, at higher ionic strength ([Na\textsubscript{2}PO\textsubscript{4}H] ≈ 100mM) we expected that changes in the interfacial environment and the bulk nucleic acid conformation would affect the ability to achieve LC orientational transitions. While others have observed LC ordering transitions with increased ionic strength, due to the formation of electric double layers\textsuperscript{33} or interactions between the LC and chaotropic anions\textsuperscript{34}, the conditions in our system are outside of the regime where these phenomena occur. Instead, we expected that increased ionic strength in the bulk aqueous phase would screen electrostatic interactions between the cationic head groups of the surfactant adsorbed at the aqueous/LC interface, allowing them to pack more tightly.\textsuperscript{29} Perhaps more importantly, a similar electrostatic screening of the anionic DNA backbone at high ionic strength promotes DNA folding into a tightly wound random coil or, if the sequence permits it, a hairpin like
Both of these phenomena inhibit the ability for the nucleobases of unbound aptamers to interact with the LC subphase and perturb the OTAB surface coverage. Consequently, we expected a decreased LC response to unbound aptamer adsorption under conditions of higher ionic strength. We observed that, for the adenosine and arginine aptamers, the fractional increase in planar area, \( f_P \), indeed decreased at higher ionic strength (Figures 4.5e, 4.5g; Table 4.1).

We also hypothesized that the subsequent LC reorientation upon aptamer-ligand binding relied on a significant decrease in the hydrophobicity of the adsorbed nucleic acid. However, a conformational change from a tightly wound random coil or hairpin structure may not involve a sufficiently dramatic change in nucleobase exposure to perturb the competitive balance for

<table>
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<th>( f_H )</th>
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<td>0.14 ± 0.14</td>
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Table 4.1 – \( f_P \) : fractional increase in planar area upon addition of aptamer; \( f_H \) : fractional increase in homeotropic area upon addition of appropriate ligand.
adsorption sites between DNA and OTAB. In fact, we observed that, at high ionic strength, ligand binding to the adenosine aptamer failed to induce a significant LC reorientation (Figure 4.5e-4.5f) while ligand binding to the arginine aptamer revealed a qualitatively similar response to that observed at lower ionic strength (Figure 4.5g-4.5h; Table 4.1). This suggested that, at $[\text{Na}_2\text{PO}_4\text{H}] \approx 100\text{mM}$, the conformational change of the adenosine aptamer upon ligand binding involved an
insignificant change in nucleobase exposure, as we would expect if the free adenosine aptamer was already in a folded (ie. hairpin) conformation prior to ligand binding. Conversely, ligand binding to the arginine aptamer still involved significant changes in nucleobase exposure at high ionic strengths, suggesting the free arginine aptamer was in a coil conformation at the increased ionic strength tested. Our structural studies, described below, are consistent with this model.

Bulk phase Förster resonance energy transfer (FRET) measurements of a dual-labeled adenosine aptamer and bulk phase circular dichroism (CD) spectroscopy provided the basis for these structural studies. With increasing ionic strength, the relative end-to-end distance \( d \) (calculated from FRET measurements as described in the Experimental Methods) decreased for both free and bound aptamer due to increased electrostatic screening (Figure 4.6a), as expected; however, this decrease was much more dramatic for the aptamer in the absence of ligand. Based on the model described above we expected a large difference in the end to end distance (\( \Delta d_{\text{low}} \)) between the free and bound aptamer at low ionic strength (ie. dramatic conformational change) and a significantly smaller difference (\( \Delta d_{\text{high}} \)) at high ionic strength (ie. subtle conformational change). Consistent with these expectations, we found experimentally that \( \Delta d_{\text{low}} >> \Delta d_{\text{high}} \).
Short end-to-end distances of nucleic acids are indicative of either a tightly packed globular coil state or a hairpin-like structure. While FRET measurements cannot distinguish between these two states, we can make inferences based on the known conformations of the adenosine-aptamer ligand complex. Past studies have shown that the adenosine aptamer forms a highly folded aptamer-ligand complex where the 5’ and 3’ tails of the DNA are adjacent, analogous to a hairpin conformation. Under the plausible assumption that the aptamer-ligand complex at low ionic strength was in such a configuration, we conclude that the free aptamer at high ionic strength was also in a hairpin conformation, since $d$ for this free aptamer was within 3% of that for the bound aptamer at low ionic strength.

CD spectroscopy measurements further elucidated the aptamer conformations. The formation of hairpin like structures in nucleic acids result in characteristic CD spectral shifts while a transition from a loose random coil to a more compact globular structure is not expected to induce significant spectral shifts (described in detail in the methods section). The CD spectra of the free adenosine aptamer revealed spectral shifts at increased ionic strength (Figure 4.6b), consistent with hairpin formation, providing further evidence that the inability for ligand binding to induce a LC reorientation at increased ionic strength was related to an insignificant change in nucleobase exposure. Furthermore, the CD spectra of the free arginine aptamer was unvarying at high and low ionic strength (Figure 4.6c), indicative of a coil structure in both cases. This was consistent with a significant change in nucleobase exposure upon ligand binding (and the associated LC reorientation) at all ionic strengths measured. We also measured the CD spectra following addition of the appropriate ligand (Figure 4.7) at concentrations $\approx 10K_d$, and observed spectral shifts indicative of
conformational changes consistent with those reported in the literature\textsuperscript{31,32}, providing evidence that ligand binding occurred for both aptamers at both ionic strengths tested.

A mechanistic summary is schematically presented in Figures 4.6d and 4.6e. When the free aptamer is in a random coil conformation (Figure 4.6d, as for the adenosine aptamer at low ionic strength or the arginine aptamer at both low and high ionic strength), with exposed nucleobases, adsorption to the surfactant laden aqueous/LC interface results in an association between the exposed nucleobases and the LC subphase, via hydrophobic interactions, inducing a transition to planar/tilted LC orientation, as described previously\textsuperscript{28}. Subsequent addition of the appropriate ligand results in a dramatic reduction in nucleobase exposure due to the formation of a highly folded aptamer-ligand complex, inducing the previously-described transition to homeotropic orientation. Conversely, when the free aptamer is already in a folded conformation (e.g., a hairpin structure, Figure 4.6e), with few exposed nucleobases (the adenosine aptamer at high ionic strength), adsorption to the surfactant laden aqueous/LC interface fails to cause a strong transition to planar LC orientation. Subsequent addition of the appropriate ligand may induce the formation of some intricate tertiary structures (e.g., G-quadruplex), but since the aptamer transitions from a weakly folded hairpin to these tertiary structures there is little change in the amount of exposed hydrophobic bases. Consequently there is a negligible change in the competitive balance between the DNA and the interfacial OTAB for adsorption sites and the LC orientation is unaffected by the addition of ligand in this case.
4.3 Conclusion

The phenomena described here establish mechanistic principles sufficient to guide the development of LC based aptasensing while also contributing to a fundamental understanding of LC anchoring. The underlying mechanisms of LC anchoring are complex and potentially involve a range of non-covalent interactions including electrostatic, coordination, and steric effects. In the current study we find that a deviation from a homeotropic LC anchoring state, associated with the interdigitation of the calamitic LC between surface bound alkyl surfactants, occurred when an amphiphilic polymer (ie. single stranded nucleic acid) effectively competed with these surfactants for adsorption sites. We provide a direct relationship between the amphiphilic nature (ie. nucleobase exposure) of the adsorbed nucleic acid and the LC anchoring. A previous study from our group provided evidence consistent with this mechanism but the direct relationship between nucleic acid structure and the LC anchoring presented here advances our understanding of how relatively subtle changes in amphiphilic adsorbates can induce LC reorientations. By employing this broadly applicable and controllable conformational change in a surface bound species to induce a predictable LC anchoring transition we illustrate the potential for highly multiplexed aptasensing applications.

This aptasensing scheme is label-free and has successfully utilized LCs as a transduction element that responds specifically to conformational changes in the aptamer upon ligand binding, fulfilling the fundamental requirements for a highly-multiplexed aptasensor. Importantly, the non-specific adsorption of free aptamer did not induce a false-positive, but in fact promoted a negative response (planar LC orientation). As described above, this is not the case in many other aptasensing strategies, forcing them to rely on efficient rinsing of non-specifically adsorbed species, a technique
which is infeasible for highly-multiplexed applications. Moreover, with our approach, multiplexed
detection of aptamer targets can be readily combined with simultaneous detection of nucleic acid
targets via hybridization. Realization of an actual LC-based aptasensor device will require a
demonstration of performance in complex media and employing micro-structured substrates as a
LC sensing platform, an area in which our lab and others have made significant progress.
While, our results at varying ionic strength may suggest that some aptamers are more limited than
others in terms of their ability to function under diverse conditions, tweaking the interfacial
conditions, such as surface charge density and surfactant composition, will allow for the rational
design of arrays for the simultaneous detection of a range of aptamer targets in a complex media
(eg. waste water, serum, etc.). Using this type of rational design and the molecular understanding
of the LC reorientation achieved here, our future work will involve extending the scope of our
approach toward a detection platform capable of label-free simultaneous multi-species detection.

4.4 Experimental Methods

4.4.1 Sample Preparation and Characterization

Preparation of self-assembled monolayers (SAMs) of octadecyltrithoxysilane (OTES) (Gelest Inc.)
was completed according to published procedures. Soda lime glass microscope slides (Corning
Inc.) were cleaned sequentially with 2% aqueous micro-90, deionized water (18.2 MΩ), and piranha
solution (30% aqueous H₂O₂ (Fisher Scientific) and concentrated H₂SO₄ (Fisher Scientific) 1:3, v/v) at
≈80°C for 1 hr. (Warning: piranha reacts strongly with organic compounds and should be handled
with extreme caution; do not store in a closed container). Following piranha cleaning, microscope
slides were rinsed with deionized water (18.2 MΩ) and dried under a stream of ultrapure N₂. A
deposition solution of n-butylamine (Fisher Scientific) and OTES was prepared in toluene (Fisher
Scientific) at 1:3:200 volumetric ratios, respectively, and warmed to 60°C. The clean and dry microscope slides were then rinsed with toluene, submerged in the warm deposition solution, and incubated for 1 hour at 60°C. Upon removal from the deposition solution, the slides were rinsed with toluene, dried under a stream of ultrapure \( N_2 \), and stored at room temperature in a vacuum desiccator. A custom-built contact angle goniometer was used to verify that the water contact angle (\( \theta_c \), measured via the static sessile drop method) of the prepared SAMs was sufficient to indicate strong homeotropic anchoring (\( \theta_c > 95^\circ \)).

OTAB (Sigma-Aldrich) laden LC films were prepared by housing the nematic E7 LC (Merck KGaA) within the pores of an electron microscopy grid (Electron Microscopy Sciences) placed onto a solid glass substrate functionalized with an octadecyltrithoxysilane self-assembled monolayer (SAM)\(^{44} \). The SAM maintained homeotropic orientation of the LC at the solid substrate. The grids were contained within silicone isolators (Grace-bio Labs, #664206) placed onto SAM-functionalized glass. A solution of OTAB in E7 was prepared at [OTAB]≈100μM. The pores of the electron microscopy grid were then filled with the LC/OTAB mixture by pipetting ≈250nL into the grid and removing the excess via capillary action. Next, the wells were filled with ≈25μL of an aqueous solution (2.5mM \( \text{Na}_2\text{PO}_4\text{H} \); Sigma Aldrich). The ssDNA adenosine aptamer (5’ACCTGGGGGAGTATTGCGGAGGAAGGT3’; Invitrogen), a ssDNA mismatch adenosine aptamer (5’ACCTGGGGGAGTATTGCGGACGGAAGGT3’; Invitrogen), or the ssRNA arginine aptamer (5’GACGAGAAGGACGGCCUGGUUCUACUAGCAGGUAGGUCACUGUC3’; Biosearch Technologies) were added by pipetting small volumes (1-2μL) of high concentration stocks (~100μM in dH\(_2\)O) into the aqueous phase to achieve a final [Aptamer] ≈ 2.5μM. Subsequent addition of the appropriate
ligand (adenosine, GMP, cytidine, thymidine, L-arginine, L-citruline; Sigma-Aldrich) (Figure 4.1a, 4.8) was also performed by pipetting small volumes (1-2μL) of high concentration stocks (≈11-17mM in dH₂O) into the aqueous phase. The LC orientation and textures were observed between crossed polarizers with an Olympus microscope (model BH2-UMA) modified for transmission mode.

![Chemical structure of control species](image)

Figure 4.8 - Chemical structure of control species

The relative end-to-end distance of dual-labeled adenosine aptamer (FAM-5’ACCTGGGGGAGTATTGCGGAGGAAGGT3’-TAMRA, Biosearch Technologies) was measured using Förster Resonance Energy Transfer (FRET) spectroscopy. The dual-labeled aptamer (100nM) was constituted in buffer at varying ionic strength ([Na₂PO₄H]≈7.8-100 mM) in the absence and presence (2mM) of adenosine. Using a fluorescence plate reader (Wallac 1420 VICTOR², Perkin Elmer) we excited the dual-labeled aptamer at λₑₓ =485nm and measured the emission intensity at λₑₓ,ₑₓ =528nm (Fₑₓ, donor emission intensity) and λₑₓ,ₑₓ =585nm (Fₑₓ, acceptor emission intensity). We used these intensities to determine the relative distance between fluorophores according to equation

4.1:
This equation is simplified from an equation that defines the absolute distance between fluorophores. In our studies this relative comparison of the distance between fluorophores is sufficient to make inferences about the nucleic acid conformations. Since our FRET pair is tethered on opposing ends of the DNA strand this relative separation between fluorophores is directly proportional to the relative end-to-end distance of the DNA.

4.4.2 Image Analysis

Polarized light microscopy images were analyzed using ImageJ (NIH Freeware) to determine the fractional increase in fractional homeotropic \( f_{H} \) (or planar \( f_{P} \)) area upon addition of target. The images were first binarized using a common threshold value that allowed for a qualitative distinction between birefringent (bright) and homeotropic (dark) regions. It is noted that this binarization did not provide a pure measure of the fractional homeotropic area, since azimuthal orientation of the LC around defects resulted in extinction. However, normalizing the fraction of dark pixels within a pore of the grid by the average fraction of dark pixels at \( c_{o} \) and at saturation accounted for the dark pixels that were not due to homeotropic LC orientation. This normalization was confirmed through qualitative inspection to verify that at \( c_{o} \) there were no homeotropic domains and at saturation the grids were in fact 100% homeotropic. Equation 4.2 was used to calculate the fractional homeotropic area at a given concentration \( (c) \), where \( f_{x} = \text{fraction of dark pixels at } c, f_{o} = \text{fraction of dark pixels at } c_{o}, \text{ and } f_{f} = \text{fraction of dark pixels at saturation}. \) To calculate \( f_{p} \) we replaced all dark pixel fractions with bright pixel fractions in equation 4.2.
4.4.3 LC Response Specificity

The specificity of the LC response was tested qualitatively through visual inspection of polarized light microscopy images and quantitatively via measuring the time dependence of $f_H$. As stated in the main text the LC reorientation for the adenosine and arginine aptamer were found to be specific to their appropriate target. Figure 4.3 shows images that supplement the plots displaying the time dependence of $f_H$ (Figure 4.1c, Figure 4.4). While we were able to consistently achieve a specific response for both aptamers under the appropriate conditions, the specificity of the adenosine aptamer was mildly sensitive to pH. At pH<7, a slight response to GMP was observed. However, this response observed to GMP was consistently less than that to adenosine. For example, in a sample that allowed for a transition to 100% homeotropic coverage upon addition of adenosine, the LC reorientation upon addition of GMP (at low pH) caused nucleation of homeotropic domains but the steady state homeotropic coverage was only ~20-40% of that observed for adenosine. This indicated a finite dissociation constant between GMP and the aptamer at pH<7 while at pH>7 the dissociation constant was so large that no LC reorientation was observed (due to minimal association of GMP-aptamer complexes). The dissociation constant likely varied with pH since guanosine is deprotonated at high pH (pKa=9$^{46}$). This deprotonation may have induced an electrostatic repulsion between the negatively charged DNA and guanosine. In contrast,
adenosine was not deprotonated at high pH and remained neutral, explaining the specificity of aptamer binding at pH>7.

4.4.4 CD Spectroscopy Analysis

Circular dichroism spectroscopy (Chirascan™-plus CD Spectrometer, Applied Photophysics) was used to probe the conformational changes that occur with varying ionic strength and upon addition of ligands. Either the adenosine or arginine aptamer was constituted in 2.5mM or 100mM aqueous Na₂PO₄H at 10µM to a total volume of 300µL. After measuring the CD spectra in the absence of ligand, 30µL of a concentrated ligand stock ([Adenosine]≈600µM, [Arginine]≈6600µM) was added directly to the sample cuvette and mixed via pipetting to achieve a [ligand] ≈10*Kₓ for both aptamers. We also measured the CD spectra of pure buffer at [Na₂PO₄H] = 2.5mM and 100mM, as well as adenosine and arginine in the absence of aptamer under both these buffer conditions. The data obtained was reported in terms of ellipticity (θ). After subtracting the buffer baseline, we converted θ to molar circular dichroism (Δε) according to equation 4.3,

\[
\Delta \varepsilon = \frac{\theta / 32.982}{C \times l}
\]  
(eq. 4.3)

where \( C \) is the concentration of nucleic acid in moles of nucleobases and \( l \) is the optical path-length. Finally, we subtracted the ligand contribution to \( \Delta \varepsilon \) on a per mole basis and applied the Savitzy-Golay algorithm to smooth the resulting CD spectra.
CD spectroscopy reports how circularly polarized light interacts with chiral molecules. As such, conformational inferences of polymeric molecules can be made by comparing experimental CD spectra to the spectra of well-known structures. CD has been extensively used for measuring conformations of proteins and nucleic acids and models have been developed to try and correlate the observed CD spectra with a well-defined structure. While these models for calculating the CD spectra of nucleic acids have been successful in some cases, a comprehensive strategy for extrapolating high resolution structures from CD spectra of nucleic acids has yet to be realized, especially when studying aptamer-ligand complexes. It is well known that base stacking, and consequently DNA sequence, is one of the major contributors to the CD spectra of nucleic acids. While this area has been well studied, the contribution of non-Watson-Crick base pairing or interactions with ligands is not as well studied, making it difficult to apply a model for structures that involve these types of interactions (i.e. aptamer-ligand complexes). For these reasons, the structural inferences we make here, involve comparing the spectra of known nucleic acid conformations to our experimental data.

In the results section we inferred from the CD spectra of the free adenosine and arginine aptamer at high and low ionic strength that the adenosine aptamer was in a random coil at low ionic strength and a weak hairpin at high ionic strength, while the arginine aptamer was in a random coil at low and high ionic strength. The CD spectra of the free adenosine aptamer upon increasing the ionic strength revealed the appearance of a shoulder at ≈210nm, a decrease in the negative peak at ≈240nm, and an increase and shift of the positive peak at ≈270nm. While the exact spectral shifts
are highly dependent on sequence, the appearance of a shoulder at ≈210nm and the shift of the peak at ≈270nm upon ligand binding were consistent with previous studies of the CD spectral changes that occur during DNA melting. Thus, we concluded that the adenosine aptamer forms a weak hairpin at this increased ionic strength. The CD spectra of the free arginine aptamer at high and low ionic strength revealed no significant spectral differences. At low ionic strength (2.5mM [Na₂PO₄H]) we expected minimal electrostatic screening and consequently a random coil configuration. As we increased the ionic strength to 100mM, it was unclear what configuration to expect since there was a potential for significant electrostatic screening, but since we observed no spectral shifts at this ionic strength we conclude that the ssRNA remained in a random coil configuration even at increased ionic strength. Furthermore, the observed spectra is consistent with others previously reported for ssRNA.

We also measured the CD spectra of these aptamers in the presence of ligand at ≈10K₀ (Figure 4.7). For both aptamers we observed dramatic shifts in the CD spectra at high and low ionic strength, consistent with previously reported crystal structures. Upon binding, the adenosine aptamer is known to undergo Watson-Crick base-pairing at its tails and form a G-quadruplex structure at its head. These types of conformational changes are known to induce large CD spectral shifts. However, the CD spectrum expected from the aptamer-ligand complex will be an average of the contributions from the double helix structure and the G-quadruplex structure, thus a theoretical spectrum of this structure would be speculative and provide little value over an empirical comparison. The spectral shifts we observed upon addition of adenosine, at low and high ionic strength, involved an increase and shift to lower wavelength in the positive peak at ≈270nm, a
decrease in the negative peak at ≈260nm, and an increased CD signal at λ<210nm. Previous CD spectral studies of DNA melting\textsuperscript{36} are consistent with the spectral shifts of the peak at ≈270nm, as mentioned above. The literature on the CD spectra of the G-quadruplex\textsuperscript{48,49} known to form for the adenosine aptamer (antiparallel)\textsuperscript{31} are consistent with our observation of a decrease in the negative peak at ≈240nm and an increase in the positive peak at ≈270nm. It is also noted that the CD spectral shifts that occur upon G-quadruplex formation are more dramatic than those that occur upon helix formation, explaining why we still see a dramatic change in the CD spectra at ionic strengths (~100mM [Na\textsubscript{2}PO\textsubscript{4}H]) where we expect that ligand binding does not induce significant Watson-Crick base pairing. The arginine aptamer revealed a decrease in the negative peak at ≈205nm upon ligand binding. This shift occurred at high and low ionic strength, consistent with ligand binding under these conditions. CD studies of RNA have revealed that an increased intensity of the negative CD peak at ≈205nm is consistent with Watson-Crick base pairing.\textsuperscript{51} The spectral shifts of the base pairing are highly dependent on sequence and usually also involve an increase in the CD peak at ≈265nm. However, the structural changes that are known to occur do not purely involve base pairing but rather involve hydrogen bonding of the bases in the binding pocket with arginine as well as base pairing in other parts of the RNA strand.\textsuperscript{32} Nevertheless, the increased intensity of the negative CD peak at ≈205nm is consistent with calculations and observations from the literature and is a good indication of ligand binding to the arginine aptamer.

4.5 References


Chapter 5: DNA Hybridization-Mediated Liposome Fusion at the Aqueous-Liquid Crystal Interface

The prominence of receptor-mediated bilayer fusion in cellular biology motivates development of biomimetic strategies for studying fusogenic mechanisms. We report an approach for monitoring receptor-mediated fusion that exploits the unique physical and optical properties of liquid crystals (LC). We use PEG functionalized lipids to create an interfacial environment capable of inhibiting spontaneous liposome fusion with an aqueous/LC interface. Then, we exploit DNA hybridization between oligonucleotides within bulk phase liposomes and a PEG-lipid monolayer at an aqueous/LC interface, to induce receptor-mediated liposome fusion. These hybridization events induce strain within the liposome bilayer, promote lipid mixing with the LC interface, and consequently create an interfacial environment favoring re-orientation of the LC to a homeotropic (perpendicular) state. Furthermore, we exploit the bi-functionality of aptamers to modulate DNA hybridization mediated liposome fusion by regulating the availability of the appropriate ligand (i.e. thrombin). Here, we outline a LC based approach for monitoring receptor (i.e. DNA hybridization) mediated liposome fusion, explore how liposome properties dictate fusion dynamics, and provide an example of how this approach may be used in a bio-sensing scheme.
5.1 Introduction

Understanding how to control bilayer fusion is fundamentally and technologically important for designing synthetic gene transfer agents, drug delivery strategies, studying biological systems, and developing diagnostic assays. In particular, *in vivo* biomimetic strategies for studying receptor-mediated fusion have played a major role in the advancement of this field. Since Rothman and coworkers first demonstrated that SNARE proteins were the minimum machinery required for inducing membrane fusion, they have been widely accepted as the most efficient fusogenic receptors. Their biological origin and prevalence in cellular membranes have inspired exploration of the mechanisms that allow SNARE proteins to work with such high efficacy. A common motif has been found among SNARE receptors that involve a bundle of four alpha-helices that associate upon recognition. The configuration of this quaternary structure induced strain to the associated lipid bilayers, initiating the fusogenic process. Several synthetic approaches that mimic this structural motif have been developed using peptides, model proteins, small molecules, and DNA in an effort to achieve efficient recognition, bilayer disruption, and content transport *in vivo*.

In particular, DNA hybridization-mediated fusion shows promise as a reductionist system both for studying fusion mechanics and as a bio-sensing strategy. Studies have shown that DNA can be anchored to lipid bilayers using DNA-lipid conjugates or sterol tethered DNA. Uni-lamellar liposomes can therefore be prepared with such tethered oligonucleotides. When two liposomes prepared with different but complementary oligonucleotides were combined, lipid mixing assays revealed bilayer fusion. A critical requirement in these assays was that membrane anchors on complementary DNA strands had to be on opposite ends of the DNA (i.e. 5’ and 3’ ends). In this configuration, DNA hybridization mimicked the configuration of the four helix bundle in SNARE
receptors, brought the two bilayers into close proximity, strained the bilayer structure, and consequently induced efficient lipid mixing and content transport. In the alternative situation where the tethers are on the same end of the DNA, the liposomes were observed to aggregate but no lipid mixing or content transport occurred, presumably due to a lack of bilayer-bilayer proximity and strain.

Studying receptor-mediated fusion in dispersed liposomes is convenient for proof-of-concept studies but has limited capacity for advancing related technologies. Alternatively, receptor-mediated fusion with planar interfaces, and in particular supported lipid bilayers, has been used for quantitative high throughput studies that elucidate cellular mechanisms related to drug discovery, medical diagnostics, and biosensor development. Supported lipid bilayers can be fabricated as spatially addressed microarrays capable of high throughput screening and have demonstrated value as a tool for studying a range of biochemical processes. Despite their success as model systems, supported lipid bilayers possess complicating factors such as interfering effects associated with the underlying solid substrate and the necessity of complex and expensive analytical instrumentation. Thus, substrates that address some of these drawbacks have significant value toward a better understanding of liposome fusion from a fundamental and technological perspective. Here, we used an alternate system for monitoring liposome fusion with planar interfaces that employed a soft hydrophobic interface (i.e. liquid crystals) for depositing lipid monolayers with surface anchored receptors (i.e. DNA). We anticipated that the hydrophobic nature of the LC substrate would minimally interfere with surface anchored components while also serving as a transduction element requiring only a simple optical set up for dynamic monitoring of fusogenic activity.
Liquid crystals (LCs) have become a valuable tool for monitoring interfacial phenomena at both solid and aqueous interfaces\textsuperscript{14}. Examples include self-assembly processes\textsuperscript{15-17}, the dynamic behavior of hydrophobic polyanions\textsuperscript{18,19}, protein binding\textsuperscript{20,21} and surfactant phase behavior\textsuperscript{22}. Studies have revealed that certain surfactants partitioned to the hydrophobic aqueous/LC interface orient calamitic LC molecules with their long axes perpendicular to the surface (homeotropic orientation)\textsuperscript{15,17}. Importantly, only surfactants with hydrocarbon chains longer than a certain threshold length were observed to induce homeotropic orientation.\textsuperscript{17} Among this class of surfactants were saturated phospholipids, which were deposited at the aqueous/LC interface via spontaneous liposome fusion.\textsuperscript{23} This spontaneous fusion was shown to be independent of lipid phase, and lipid/surfactant mixtures were employed to control the relative density of lipids within a monolayer at the aqueous/LC interface. Thus, spontaneous fusion of liposomes with LC interfaces has been well characterized and is readily monitored using the optical effects associated with LC re-orientation.

More recent studies have achieved specific control over interfacial LC orientational transitions by exploiting conformational changes in adsorbed biomolecules\textsuperscript{18,19}, localizing polar molecules via coordination interactions\textsuperscript{24}, or mediating surfactant deposition with biomolecules\textsuperscript{20}. In one example in particular, liposome fusion was controlled at protein decorated interfaces.\textsuperscript{20} Liposomes were prepared with biotinylated phospholipids and introduced to a streptavidin laden LC interface. Streptavidin inhibited spontaneous fusion of undecorated liposomes with the interface while also acting as a probe to detect biotin in dispersed liposomes. When biotin bound to streptavidin at the interface, liposome fusion was initiated, and a transition to homeotropic orientation was observed. This example provided a proof-of-concept for using specific binding events to deposit lipids at an
aqueous/LC interface. A more universal (and potentially multiplexed) approach requires the incorporation of membrane-anchored receptors that are compatible with a generalizable interfacial environment for inhibiting spontaneous liposome fusion.

The aim of our current study was to design a system for using biomolecular interactions to induce liposome fusion. We utilized PEG-lipid laden aqueous LC interfaces, which are inherently resistant to non-specific adsorption. We hypothesized that these interfaces would inhibit spontaneous liposome fusion and provide sufficient resistance to non-specific protein adsorption to enable the detection of specific protein binding events. Oligonucleotides were anchored to bilayers within liposomes and at the aqueous/LC interface and served as receptors for mediating liposome fusion. Using this experimental system our aims were to (i) observe and characterize DNA hybridization-mediated liposome fusion via a LC reorientation, (ii) explore the liposome properties that dictate the kinetics associated with these fusion events, and (iii) demonstrate the applicability of this approach in a detection scheme utilizing aptamers.

5.2 Materials and Methods

5.2.1 Materials

Lipids (i.e. (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]-1000) (ammonium salt)) DSPE-PEG1k; (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]-2000) (ammonium salt)) DSPE-PEG2k; (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]-5000) (ammonium salt)) DSPE-PEG5k; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)) were purchased from Avanti Polar Lipids Inc. Oligonucleotides (5’chol-DNA: CHOL – 5’ TCC GTC GTG CCT TAT TTC TGA TGT CCA AAA CCA ACC ACA
3'; 3' chol-DNA: 5' GTT GGT TTT GGA CAT CAG AAA TAA GGC ACG ACG GA 3’ - CHOL, and aptamer: 5’ GTT TGG TGT GGT TGG TTT 3’) were purchased from IDT Technologies. Thrombin from human plasma (≥1000 NIH units/mg) was purchased from Sigma-Aldrich and suspended in aqueous buffer at concentrations ranging from 3-10 μM as verified by absorption measurements at λ=280nm (i.e. Nanodrop 2000). A liquid crystal mixture, E7 (a mixture of three cyano-biphenyls and a cyano-terphenyl), was purchased from Merck Kga. Aqueous buffers were prepared from powder stocks of Tris-HCl (Fisher) and NaCl (Fisher) constituted at the proper concentrations in deionized water (18.2 MΩ-cm) and adjusted to pH ~8.5 by addition of 0.1M NaOH. Octadecyltriethoxysilane was purchased from Gelest Inc. and deposited onto soda-lime glass microscope slides (Fisher) according to the procedure outlined below. Electron microscopy grids (CP-100) were purchased from Electron Microscopy Sciences. Silicone isolators were purchased from Grace Bio Labs and were used as wells to contain the aqueous phase above the LC interface.

5.2.2 Liposome preparation

Liposomes were prepared by first dissolving a known mass of the appropriate lipid in chloroform. Chloroform was then removed by rotary evaporation at ~55 °C with a rotation speed of 200-250 rpm to obtain a homogenous lipid film. The film was subsequently solvated in an aqueous buffer (10 mM Tris, 100 mM NaCl, pH ~8.5) at [lipid]~6.8 mM and incubated in a water bath at ~70 °C for 30-60 m. Once lipids were completely dispersed in aqueous buffer, we diluted the lipid solution to the desired concentration and added the applicable DNA strands at the appropriate concentrations. The lipid/DNA mixture was incubated at ~70 °C for 30-60 m to allow sufficient time to incorporate into the lipid bilayer. Finally, we sized the vesicles using a membrane extruder (Avanti Polar Lipids Inc.) with membrane pore sizes (D_pore) of 50, 100, or 400 nm. Liposome diameters were subsequently
verified using photon correlation spectroscopy. DSPE-PEG micelles were always prepared at the same concentration by mixing DSPE-PEG (and 3’ chol-DNA when applicable) with aqueous buffer (10 mM Tris, 100 mM NaCl, pH ~8.5) to a final [DSPE-PEG]=40.2µM ([3’chol-DNA]=0.85 µM) and vortexing the mixture for 30-60 s.

5.2.3 LC film Preparation

Preparation of self-assembled monolayers (SAMs) of octadecyltrithoxysilane (OTES) was completed according to published procedures25. Briefly, glass slides were cleaned sequentially with 2% aqueous Micro-90 detergent, deionized water (18.2 MΩ-cm), and piranha solution (30% aqueous H₂O₂ (Fisher Scientific) and concentrated H₂SO₄ (Fisher Scientific) 1:3, v/v) at ≈ 0 °C for 1 h. (Warning: piranha reacts strongly with organic compounds and should be handled with extreme caution; do not store in a closed container). Piranha-cleaned microscope slides were then rinsed with deionized water (18.2 MΩ-cm) and dried under a stream of ultrapure N₂. Toluene, OTES, and n-butylamine (Fisher Scientific) (Fisher Scientific) were mixed at 200:3:1 volumetric ratios, respectively, and warmed to 60 °C. Clean and dry microscope slides were rinsed with toluene, submerged in the warm deposition solution, and incubated for 1 h at 60 °C. Following this incubation, the slides were rinsed with toluene, dried under a stream of ultrapure N₂, and stored at room temperature in a vacuum desiccator. The water contact angle (θ_C, measured via the static sessile drop method) of the prepared SAMs was verified to be > 95° (indicative of strong homeotropic anchoring) using a custom-built contact angle goniometer.

Aqueous/LC interfaces were prepared by housing LC films within the pores of an electron microscopy grid. The grids were initially placed onto a solid glass substrate functionalized with an
OTES self-assembled monolayer (SAM) and contained within silicone isolators. The SAM served to maintain homeotropic orientation of the LC at the solid substrate. The pores of the electron microscopy grid were then filled with E7 by pipetting $\approx 250 \text{ nL}$ into the grid and removing the excess via capillary action. Next, the wells were filled with $\approx 25 \text{ μL}$ of an aqueous solution. When depositing DSPE-PEG micelles to the interface, aqueous buffer containing the appropriate DSPE-PEG micelles were added during this initial introduction of the aqueous phase. Liposomes were subsequently added two minutes after the initial introduction of the aqueous phase by pipetting an additional 25 μL of the desired liposome mixture, mixing the solution in the wells by pipetting, then removing 25 μL to re-establish the planar air-water interface that allows for efficient imaging (note: curvature of the air-water interface caused reflections that distorted polarized light microscopy images). The LC orientation and textures were dynamically monitored between crossed polarizers using a custom built transmission microscope.

5.2.4 Image Analysis

Images obtained from polarized light microscopy (PLM) were analyzed using customized algorithms in the Mathematica programming environment. The region of interest was first selected using an image convolution function to find the center of the electron microscopy grid, and a constant grid diameter to select the region of interest for analysis. Next, images were binarized to obtain the fraction of dark pixels in a given image. However, this fraction of dark pixels did not directly correlate with homeotropic coverage since in-plane (i.e. azimuthal) alignment of LC molecules around point defects resulted in extinction at angles parallel or normal to the incident polarization of light. Since the surface area of LC film that experienced this extinction in our system was relatively constant we were able to circumvent this artifact by using a normalization approach. We
obtained the fractional increase in homeotropic coverage ($\Delta f_H$) as a function of time within a series of images according to the following equation:

$$\Delta f_H = \frac{f_t - f_o}{1 - f_o}$$  

(eq. 5.1)

where $f_o$ was the fraction of dark pixels in a qualitatively planar image from the same time series and $f_t$ was the fraction of dark pixels at time=t.

5.3 Results and Discussion

5.3.1 Creating a steric barrier to liposome fusion

While past studies have characterized the phase behavior of PEG-lipids at aqueous/LC interfaces\textsuperscript{22}, the extent to which these interfaces inhibit non-specific adsorption had not previously been determined. Thus, our first step involved characterizing DSPE-PEG (and 3’chol-DNA) laden aqueous/LC interfaces. When micelles comprised of PEG-functionalized DSPE (DSPE-PEG) and 3’chol-DNA were introduced to the aqueous phase in contact with the LC, we observed no change in the LC orientation from the native planar orientation observed at undecorated aqueous/LC interphases. This was true for PEG molecular weights ranging from 1-5 kDa (Figure 5.1a,c,e). Thus, under the assumption that the micelles fused with the bare LC interface, one would conclude that the presence of a DSPE-PEG/3’chol-DNA monolayer was insufficient to cause a re-orientation of the LC phase. Another less likely hypothesis, however, was that the micelles did not fuse with the LC interface. As described below, the response of the interface to subsequent changes was used to test these hypotheses.
In particular, the ability of DPPC liposomes to fuse with the aqueous/LC interface was found to be sensitive to the presence (or absence) of a steric barrier (i.e. PEG). When DPPC liposomes were added to the aqueous phase in contact with an LC interface, a transition to homeotropic orientation occurred due to spontaneous fusion and deposition of lipids at the interface (Figure 5.2). The driving force for this spontaneous fusion was the relative instability of these aggregate structures. DPPC, like most other saturated phospholipids, has a packing factor close to unity\textsuperscript{26}. These packing factors favor a planar lamellar configuration but form spherical bilayer aggregates in bulk solution to minimize the energy costs associated with open ends in planar lamellae.\textsuperscript{27} When a hydrophobic
interface was accessible, the lipids partitioned to the interface and assembled into a favorable planar monolayer configuration without ends that were exposed to an aqueous environment.

However, if there was a steric barrier at the interface, the proximity of the liposomes to the hydrophobic interface may be insufficient to induce spontaneous disruption of the bilayer structure. In fact, this was exactly what we observed, as illustrated in Figure 5.3, where planar LC orientation was maintained for at least 1h after adding DPPC liposomes to the aqueous phase in contact with a DSPE-PEG/3’chol-DNA laden interface. The same behavior was observed when 3’chol-DNA was not included in the micelles used to pre-load the interface, indicating that DSPE-PEG alone was sufficient to inhibit spontaneous liposome fusion. Importantly, this result verified that the DSPE-PEG micelles did in fact spontaneously fuse with the LC interface since their presence dramatically modified the interfacial fusion behavior of the DPPC liposomes. Thus, we successfully created an interfacial environment capable of inhibiting spontaneous liposome fusion.

Figure 5.2 – Protein adsorption: The fractional increase in homeotropic coverage plotted against time when DPPC liposomes are added at 0min (both black and red curves) and thrombin is added at 2 min (red curve).
5.3.2 Overcoming the steric barrier with DNA hybridization

Next, we sought to utilize receptor binding (i.e. DNA hybridization) to overcome the steric barrier associated with liposome fusion to the PEG-laden LC interface. Receptor-mediated fusion involves two key steps: (1) recognition and (2) destabilization of the bilayer structure. We hypothesized that if 5’chol-DNA was anchored to liposomes and introduced to a LC interface laden with complementary 3’chol-DNA, then recognition (or docking) would occur between the liposomes and the interface. To further increase the likelihood of destabilization, we used a design where the complementary DNA strands had tethers that partitioned to the same interface after hybridization (Figure 5.4A-B) and induced strain in the liposome bilayer structure. Using this approach, we successfully demonstrated receptor-mediated fusion between liposomes and the aqueous/LC interface. Specifically, a transition to homeotropic anchoring was observed (Figure 5.4D, 5.5A-C) upon addition of DPPC/5’chol-DNA liposomes to a DSPE-PEG1k/3’chol-DNA laden aqueous/LC interface. To verify that the lipid mixing was in fact receptor-mediated, we performed control experiments where the DNA was omitted from either the liposome or the planar interface. In both
cases we found that no lipid mixing was observed (i.e. the LC orientation remained planar) (Figure 5.4D, 5.5D-I).

Figure 5.4 - DNA hybridization mediated fusion: (A,B) schematic representation of DNA hybridization mediated fusion where DPPC/5′chol-DNA liposomes are added above a DSPE-PEG1k/3′chol-DNA laden LC interface (A). DNA hybridization deposits DPPC at the interface (B) to induce homeotropic LC orientation. (C) DNA sequences used (D) The fractional increase in homeotropic area ($\Delta f_H$) observed when adding liposomes ([DPPC]=4.1mM; [5′chol-DNA]=1.6μM; $D_{pore}=50$nm) 2 minutes after the initial introduction of the aqueous phase. The black curve indicates that both 5′chol-DNA and 3′chol-DNA were present in their appropriate location while the red curve is the response when 3′chol-DNA was left out and the blue curve is the response when 5′chol-DNA was left out. Error bars represent the standard error associated with the experimental data.
Consistent with the proposed mechanism, when we varied the molecular weight of the PEG group tethered to DSPE-PEG at the interface, we observed a transition to homeotropic anchoring only when the PEG molecular weight was ≤2kDa (Figure 5.1). Importantly, for each PEG MW tested, no liposome fusion was observed in the absence of 5’chol-DNA. These results suggested that when PEG groups were too large, the steric barrier was too great, consequently hindering either DNA hybridization or bilayer rupture. Also, when we varied the liposome size by extruding them through membranes with various pore sizes we observed no significant change in the fusion kinetics with liposome diameter (Figure 5.6). This suggested that given the fast kinetics observed, an increase in the liposome mobility (smaller liposomes) or available lipids per liposome (larger liposomes) had an insignificant effect on the rate of lipid mixing.
To better understand the effects of DNA coverage on receptor-mediated fusion kinetics we systematically varied the 5’chol-DNA content of the liposomes (see Figure 5.7). Previous studies have shown that an increased coverage of DNA on unilamellar liposomes increased the kinetics of fusion between dispersed liposomes possessing complementary oligonucleotides. However, these studies addressed only a relatively narrow regime of DNA coverage and did not address the potential inhibition of fusion that could occur due to electrostatic repulsion between liposomes at high DNA coverage. As shown in Figure 5.7, we found that the rate of fusion was non-monotonic with respect to the DPPC:5’chol-DNA ratio. In the regime of high DNA content we observed no indication of fusion (i.e. change in LC tilt angle) on the time scales of the experiment, indicating complete inhibition of fusion. In this case, the electrostatic repulsion might have been so great that complementary DNA strands were not able to hybridize. At low 5’chol-DNA coverage we also saw a slower rate of lipid mixing, but the fusion was not completely inhibited (Figure 5.7A). Here, we
speculated that hybridization was still able to proceed but, because of the low surface density of DNA, the probability of successful DNA targeting and hybridization was significantly decreased. These experiments provided insight into how electrostatics and DNA density were dictating the fusion dynamics in our system and provided us with an optimal range to use for maximizing fusion kinetics.

5.3.3 Molecular sensing using DNA hybridization-mediated fusion

Finally, we performed experiments that demonstrated how this type of DNA hybridization mediated-fusion could be exploited for molecular sensing. In particular we incorporated aptamers into the detection scheme so we could detect the presence of the protein thrombin. Aptamers are nucleic acids that are synthetically evolved using the SELEX (systematic evolution of ligands by exponential enrichment) process to bind to a specific target molecule (e.g. small molecules, antibodies, proteins)\textsuperscript{28,29}. They have become attractive as probes for multiplexed based assays due to the high sensitivity and specificity with which they bind to their respective target\textsuperscript{30}. Since

Figure 5.7 - 5′chol-DNA coverage: (A) The fractional increase in homeotropic coverage observed at varying DPPC:5′chol-DNA ratios. (B) The effective rate constant ($k_H$) which represents the inverse of the time required to reach 50% homeotropic coverage (upper limit=60m) as interpolated from the data. (note: [DPPC]=4.1mM; [5′chol-DNA]=1.6μM; $D_{pore}=400\text{nm}$)
aptamers are nucleic acids, studies have shown that they possessed a degree of bi-functionality since they bound to complementary DNA strands, via hybridization, and appropriate molecular targets. Often, aptamers bound with a stronger affinity to their respective ligand then to a complementary DNA sequence. We exploited this phenomenon to control the DNA hybridization mediated fusion described above by the presence of the appropriate ligand.

The detection scheme is schematically illustrated in Figure 5.8. Liposomes were prepared with 5’ chol-DNA and aptamer. (B) When these liposomes are added to a DSPE-PEG1k/3’ chol-DNA laden LC interface fusion is inhibited since the aptamer blocks DNA hybridization. (C) If the liposomes are mixed prior to thrombin addition, (D) it bound to the aptamer causing it to disassociate from 5’ chol-DNA and (D) allowed DNA hybridization mediated fusion to proceed. (E) oligonucleotide sequences used.

The detection scheme is schematically illustrated in Figure 5.8. Liposomes were prepared with 5’ chol-DNA and exposed to a complementary aptamer sequence that bound to the protein thrombin. In the absence of thrombin the aptamer was hybridized to 5’ chol-DNA (Figure 5.8A),
and effectively blocked hybridization between the liposomes and surface-anchored DNA strands (Figure 5.8B). However, when liposomes were exposed to sufficiently high concentrations of thrombin (Figure 5.8C), the aptamer was expected to dissociate from 5’chol-DNA due to competitive binding with thrombin, freeing the liposome-anchored 5’chol-DNA to hybridize with surface anchored 3’chol-DNA and promoting fusion (Figure 5.8D). Thus we postulated that the exposure of liposomes prepared with 5’chol-DNA and aptamer to a DSPE-PEG 3’chol-DNA laden interface would fail to induce a transition to homeotropic orientation. But such a transition was hypothesized to occur in the presence of thrombin.

Figure 5.9 summarizes the results of experiments testing these hypotheses as a function of thrombin concentration. Specifically, a transition to homeotropic orientation was observed only when DPPC/5’chol-DNA/aptamer liposomes were added to a DSPE-PEG1k/3’chol-DNA laden aqueous/LC interface in the presence of sufficiently high concentrations of thrombin. When the

Figure 5.9 - Thrombin Dose-Response: (A) The fractional increase in homeotropic area ($f_H$) observed when adding DPPC/5’chol-DNA/aptamer liposomes a priori incubated with varying concentrations of thrombin to a DSPE-PEG1k/3’chol-DNA laden interface (B) The fractional increase in homeotropic area 60 minutes after the introduction of liposomes ($f_{H,60}$) plotted against thrombin concentration. (note: [DPPC]=1.36mM; [5’chol-DNA]=[aptamer] =0.85μM; $D_{pore}$=100nm)
thrombin concentration was less than ~110 nM, no increase in homeotropic coverage was observed after ~60 min, while at concentrations in the range 221–831 nM we saw a transition to nearly 100% homeotropic coverage. Dose-response measurements were taken for up to ~1 h after addition of liposomes and no increase in homeotropic coverage was observed at low thrombin concentrations. However, when monitoring this system for longer times (>4hrs) we observed a slow transition to homeotropic even in the absence of thrombin. We hypothesize that this behavior was due to the dynamic behavior of DNA duplexes, in particular to the fact that the aptamer and 5’chol-DNA existed in dynamic equilibrium. Thus, for short periods of time, and at relatively low probability, the 5’chol-DNA was unblocked by aptamer, creating a small but finite probability for 5’chol-DNA/3’chol-DNA hybridization. The cumulative effect of these low probability events were observed because once they occur, they are essentially irreversible since the lipids used in our study had an effectively insurmountable energy barrier to desorption. However, the rate of these “false negative” events was sufficiently low to monitor the specific fusogenic activity mediated by aptamer-ligand binding.

5.4 Conclusions

While LC interfaces have previously been designed to detect specific proteins, a robust detection scheme that combines protein resistant surfaces with protein recognition-mediated LC orientational transformations has yet to be realized. Previous studies have shown that macromolecular proteins readily adsorbed to the aqueous/LC interface, disrupted the interfacial structure associated with homeotropic orientation, and inhibited specific protein detection. Oligo-ethylene glycol laden aqueous/LC interfaces reduced non-specific protein adsorption, consequently inhibiting the proteins’ ability to disrupt the homeotropic orientation associated with surfactant laden interfaces. Here, we employed similar interfacial conditions to create protein-resistant
surfaces within a robust detection scheme. To illustrate this point, we show that when thrombin was added during DPPC liposome fusion, protein adsorption to the interface inhibited the extent to which DPPC was able to induce a transition to homeotropic orientation (Figure 5.2). Thus, in our detection scheme, if our surfaces had no resistance to non-specific protein adsorption, we would not have been able to observe a transition to homeotropic orientation. The observation of nearly 100% homeotropic coverage, even in the presence of relatively high concentrations of thrombin (Figure 5.9A), was evidence of interfacial conditions that not only inhibited spontaneous liposome fusion but also inhibited the non-specific adsorption of proteins. While decreased fusion kinetics were observed at thrombin concentrations >800nM (likely due to thrombin interfering with the fusion dynamics), a complete transition was still observed, suggesting that the PEG sufficiently inhibited non-specific protein adsorption even at high thrombin concentrations. Thus, we have demonstrated that DNA hybridization mediated fusion at protein resistant surfaces was exploited for detection of macromolecular proteins. Since the materials required for these experiments were relatively inexpensive (requiring only a simple optical set-up) and responded rapidly ($t_{50\%} < 1\text{min}$), this approach also shows promise for high throughput screening of fusogenic receptors and aptamer-based biosensing. Future work will involve extending this approach toward detecting other types of receptor mediated liposome fusion (e.g. SNARE, small molecule) and further optimizing conditions (e.g. PEG size, DNA:aptamer ratio) for advanced aptamer based bio-sensing applications (e.g. detection in complex media).

5.5 References


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Appendices

A. Platform Development

A.1 Results and Discussion

The improved wettability of homeotropic alignment layers as achieved through the use of mixed SAMs (Chapter 2) facilitated the design of a platform for multiplexed detection at the aqueous/LC interface. In the detection schemes described above (Chapters 3-5), the LC film is supported in an electron microscopy grid and in contact with an aqueous phase. Control of the film thickness in this platform was achieved by exploiting capillary interactions to maintain a uniform and stable film where the LC layer adopted a thickness equal to the height of the grid.\(^1\) A more robust and reproducible approach involved stabilizing a continuous LC film in a micro-structured environment.\(^2,3\) In this scenario, an LC material infiltrated the entire array, filled the space between micro-pillars, and capillary forces stabilized the LC film at a thickness equal to the height of the micro-pillars. Thus, we designed a platform that mimicked the geometry of previously employed

![Figure A.1 – A platform for detection at the aqueous/LC interface: (a) Schematic of the micro-structured platform. (b) Bright-field (or (c) PM images of the platform prepared on (b) amine functionalized or (c) unmodified glass. PM images of the air/LC interface housed in a platform (d) prepared with insufficient O\(_2\) plasma cleaning; thus residual SU-8 resulted in a failed silane deposition or (e) successfully modified with a LC anchoring surface to induce homeotropic orientation at the solid/LC interface. (f) Proposed procedure for preparation of a multiplexed platform.](image-url)
electron microscopy grids, but with gaps at the corners of the pores of the grid. This design allowed
for deposition of a continuous LC film but with a large “viewing area” for the observation of LC
textures. Furthermore, we established a procedure that allowed for fabrication onto glass
substrates

A schematic of the proposed design is shown in Figure A.1a. SU-8 photoresist was employed
for fabrication onto soda-lime glass functionalized with amine terminated SAMs (N-(6-aminohexyl)
aminopropyl-trimethoxysilane). Amine terminated SAMs have previously been shown to improve
adhesion of SU-8⁴, presumably due to reaction of the epoxide moieties of SU-8 with the
nucleophilic surface amine. We indeed observed improved adhesion using amine terminated SAMs.
For example, substrates fabricated onto pure glass exhibited poor adhesion (Figure A.1c), but when
glass functionalized with an amine SAM was used, adhesion improved dramatically (Figure A.1b).
To deposit a LC anchoring surface in the area in between the substrate features, O₂ plasma and UV-
ozone cleaning of the substrates were completed to remove residual SU-8 and activate surface
hydroxyls. When a purely C18 SAM was deposited, the LC oil did not readily infiltrate the micro-
structured substrate. However, when a mixed SAM (χC₁₈=0.26 ) was utilized the LC readily infiltrated
the micro-structured substrate and was stable. The LC film created when using a mixed SAM was
stable even when in contact with an aqueous phase, demonstrating the applicability of the mixed
SAM approach discussed above. The success of SAM deposition was verified through contact angle
and LC alignment measurements (Figure A.1d,e).

These fabrication procedures enable the design of an array for multiplexed detection at the
aqueous/LC interface. A proposed scheme for multiplexed detection is outlined in Figure A.1f that
utilizes site specific DNA spotting to isolated LC films. Realization of this approach requires further
optimization (e.g. photoresist chemistry, feature size) of the lithographic procedures used to
prepare the micro-structured substrates, testing of the desorption rate of nucleic acids from
cationic surfactant laden interfaces, and incorporation of micro-structured substrates into a robust
flow cell environment.

A.2 References


B. Molecular Combing of Oligonucleotides Induces Secondary Structure Specific Azimuthal Liquid
Crystal Orientation

B.1 Introduction

Recent studies have revealed that azimuthal LC orientation can be controlled using sheared or combed DNA.\textsuperscript{1,2} In the later approach, lambda phage dsDNA combed onto a GPTMS (i.e. \([3\text{-Glycidoxypropyl}]\) methyltriethoxysilane) self-assembled monolayer revealed azimuthal LC orientation \(\sim 30^\circ\) from the combing direction. When denatured lambda phage DNA (i.e. ssDNA) was instead combed onto the surface, azimuthal LC orientation was parallel to the stretching direction. Thus, azimuthal LC orientations were used to distinguish between ssDNA and dsDNA, illustrating the potential for DNA based biosensing. The underlying GPTMS SAM promoted exquisite sensitivity since it possessed low azimuthal anchoring energy, allowing for microscopic perturbations induced by dsDNA to propagate over macroscopic distances. For example, it was shown that one dsDNA molecule could align upwards of \(10^{14}\) LC molecules, a sensitivity approaching single molecule detection.

This ability to detect specific DNA conformations (i.e. ssDNA \(\sim vs\) dsDNA) via azimuthal LC orientation shows promise as an approach for a highly sensitive and selective detection strategy. However, in order for this detection scheme to be exploited as an assay, short immobilized DNA (i.e. oligonucleotides) must first be identified via azimuthal LC orientation. The lambda phage DNA utilized in the initial proof-of-concept study described above was \(\sim 48.5\) kbps in length, while typical probes for detecting DNA hybridization (e.g. via PCR) are <100 bases long\textsuperscript{3}. Historically, DNA stretching and molecular combing experiments have utilized longer DNA strands (kbp) to study the physics associated with this phenomenon.\textsuperscript{4,5} Therefore, it was unclear whether the magnitude of
random molecular motion that occurs during molecular combing would inhibit the ability for shorter DNA probes to be efficiently combed. In order to test whether azimuthal LC orientations can be exploited to detect DNA hybridization, experiments were proposed to (1) demonstrate the ability of oligonucleotides to induce azimuthal LC orientation, (2) evaluate the sensitivity with which ssDNA can be distinguished from dsDNA, and (3) employ a microfluidic device for combing DNA.

B.2 Materials and Methods

Self-assembled monolayers of glycidoxy-propyl trimethoxysilane (GPTMS) were prepared using a liquid phase deposition approach. Borosilicate glass slides were sequentially cleaned with 2% aqueous micro-90 surfactant, deionized water (18.2MΩ), and piranha (30% aqueous H₂O₂ (Fisher Scientific) and concentrated H₂SO₄ (Fisher Scientific) 1:3, v/v) at 80°C for 1 hr. (Warning: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store in closed container). Following piranha cleaning the slides were rinsed with deionized water, dried under an ultrapure N₂ stream, and cleaned using a UV-ozone exposure for ~1 hr. The cleaned slides were then placed in a deposition solution containing 1-2% GPTMS in toluene for 10-60 min at room temperature. After incubation, the slides were rinsed with toluene, dried under an ultrapure N₂ stream, and stored in a vacuum desiccator. Efficient monolayer deposition was verified using the static sessile drop method on a custom built contact angle goniometer (θC ≈ 52°).

Oligonucleotides were deposited onto GPTMS modified substrates by exploiting the fact that amine groups react readily with epoxides at elevated pH. Amine modified DNA (“NH-probe”, Invitrogen) was dispersed in 1X PBS (pH > 8.0) at ~30nM. GPTMS modified substrates were then immersed in the DNA solution and incubated overnight to allow sufficient time for the amine groups to react
with the surface immobilized epoxide groups. After incubation the slides were rinsed with water and subsequently placed in 1X PBS with or without complementary DNA (“target”, Invitrogen). The solution without target served as the ssDNA “control” while the solution with target was used to test the ability to recognize DNA hybridization at the surface using azimuthal LC orientation. The substrates were incubated in solution for ~1hr and subsequently removed in a controlled direction and at a controlled speed (10mm/min). Finally a LC cell was made using a homeotropic alignment layer (OTES) and the DNA functionalized substrate. The LC orientation was then monitored between crossed polarizers using a custom built transmission microscope.

Alternatively, a microfluidic channel was used to control the combing direction. In this approach, NH-probe (~30nM) was first introduced to the flow cell and incubated overnight. Next, the aqueous solution was withdrawn from the flow cell, effectively introducing an aqueous/air interface within the flow cell environment. As this interface traveled through channel, ssDNA was combed perpendicular to the air interface. Once the air interface completely passed through the flow cell, the cell was disassembled and the DNA functionalized substrate was used to make a LC cell with an opposing homeotropic alignment layer (OTES).

Polarized light micrographs were analyzed to find the most probable extinction angle. Images were taken with the stretching direction rotated at some angle (θ) from the analyzer. When the azimuthal (in-plane) alignment of the LC was parallel to either the polarizer or the analyzer, extinction occurred. In order to characterize the probability of extinction at a given angle we defined the light intensity as a function of θ:

\[ I_r = \sin^2(2\theta) \]  
(equation B.1)
where $I$, was the normalized intensity and $\theta$ was the angle between the analyzer and the azimuthal
director. Thus when the azimuthal director was aligned with the stretching direction, the intensity
was minimized when $\theta$ was 0° or 90°. To find the probability for extinction at a particular angle, $\theta$,
we used a custom algorithm written in the Mathematica environment to find a correlation
coefficient between the intensity profile of every pixel within a series of images taken at varying $\theta$
and the theoretical profile associated with equation B.1. The correlation coefficient was maximized
when the azimuthal orientation was parallel with the analyzer (i.e. extinction) allowing us to define
the azimuthal director. Since this was done for every pixel we counted the number of pixels with a
particular extinction angle and divided this by the total pixel count to find the probability for
extinction at a particular angle ($p_{ex,\theta}$). Furthermore, when a gaussian function was fit to the resulting
probability distribution the average angle at which extinction occured ($\mu_\theta$) and its associated
variance ($\sigma_\theta$) could be determined according to:

$$p_{ex,\theta} = \frac{1}{\sigma_\theta 2\pi}Exp \left[ \frac{(\theta - \mu_\theta)^2}{2\sigma_\theta^2} \right] \quad \text{(equation B.2)}$$

Equation B.2 was fit to the probability distributions (from $\theta=0^\circ$-$45^\circ$) that resulted from image
analysis to find $\mu_\theta$ and $\sigma_\theta$, which were used to evaluate the sensitivity associated with varying target
concentrations.

A similar approach was used to find the extinction angle when ssDNA was stretched within a
microfluidic environment. First, a polynomial was fit to the flow profile $f(x)$ (Figure B.3B), where $x$
was the cross sectional channel position, of the receding air/water interface within the microfluidic
channel (Figure B.3C). The derivative of this polynomial was then used to define the stretching
direction. We used the reasonable assumption that at the peak of the flow profile the DNA was
stretched parallel to the channel walls (i.e. 0°) and at the edge of the channel the DNA was stretched 45° from the channel wall. Thus when we normalized the derivative of the polynomial ($f'_{\text{norm}} = f'(x)/f'(0)$) we could define the stretching direction as $f'_{\text{norm}} \times 45°$. The relative intensity ($I_r$) due to azimuthal orientation within a flow channel was then defined as:

$$I_r = \sin^2(2(f'_{\text{norm}} \times 45° + \theta))$$

Thus, when the azimuthal director was aligned with the stretching direction a minimum in $I_r$ occurred at the “peak” of the flow profile, which roughly correlated with the center of the microfluidic channel.

B.3 Results and Discussion

In fact, it was observed that short immobilized DNA oligonucleotides are capable of inducing azimuthal LC orientation when combed onto a solid substrate. When ssDNA was immobilized and combed on a GPTMS substrate, we consistently observed azimuthal LC orientation parallel with the stretching direction (Figure B.1a,b,e). However, when a complementary DNA strand was added to the aqueous phase prior to combing, DNA hybridization occurred with the surface immobilized NH-probe, and the azimuthal director was found to be ~30° from the stretching direction (Figure B.1c,d,e). It was clear from these measurements that combed oligonucleotides were capable of inducing azimuthal LC orientation. To our knowledge, this was the first example of molecular combing of polymers on this length scale (contour length<100nm). The lack of previous reports using short polymers was presumably due to an insufficient analytical technique for measuring their elongation. Thus, we demonstrated the first example of an analytical technique that measured the elongation of polymers (via molecular combing) with short contour lengths and provided a potential path forward for a DNA based biosensor scheme.
In order to better understand the azimuthal anchoring associated with dsDNA and ssDNA we systematically varied the concentration of target DNA. By varying the bulk concentration of target DNA we effectively varied the ratio of ssDNA:dsDNA present at the interface. For example, at low target concentration there could have been a high ssDNA:dsDNA ratio if there was insufficient DNA available to reach a high surface coverage of dsDNA. However, at high [target] we might have also seen a high ssDNA:dsDNA ratio since there could have been a large amount of non-specifically adsorbed (and un-hybridized) target DNA. In fact, within the concentration range that was tested (0.001 – 10 nM) we saw extinction angles of ~30° only at concentrations ≈1pM. At higher concentrations the most probable extinction angle was somewhere between 0-30°. Since the extinction angle did not go to zero at higher target concentrations (>0.1nM), we suspected that there was heterogeneity within the surface. This preliminary result suggested that the anchoring transition between purely ssDNA and purely dsDNA was continuous⁸ rather that the discontinuous transitions previously observed for homeotropic to planar alignment⁹. Despite the heterogeneity

Figure B.1 - Oligonucleotide induced azimuthal LC orientation: Polarized light micrographs of LC cells composed of OTES and either (a-b) ssDNA or (c-d) dsDNA functionalized slides there the DNA stretching axis is rotated at either (a,c) 0° or (b,d) 30° from the analyzer (θ). (e) The probability of extinction at p_{ex,0} plotted against θ for either ssDNA (blue curve) or dsDNA (red curve).
observed at elevated concentrations, we were able to achieve sensitive detection (1pM) of dsDNA via an azimuthal LC orientation. Improved surface functionalization strategies (e.g. better GPTMS SAMs and higher probe DNA coverage) should reduce the non-specific adsorption at high [target], effectively increasing the dynamic range.

In an effort toward developing a more robust sensor strategy we completed the molecular combing of short oligonucleotides within a microfluidic environment and established the basic principles for analyzing the resultant polarized micrographs. Previous reports have shown that DNA can be elongated using a moving meniscus within a microfluidic channel.\textsuperscript{10} We designed a similar device capable of combing immobilized DNA. This device needed to be capable of disassembly after molecular combing without disrupting the surface immobilized DNA. To meet this requirement, we used silicone rubber as the walls of our flow cell. Silicone rubber sticks to glass surfaces via non-covalent adhesion forces sufficient to form a water tight seal, but can often be removed after use.

Figure B.2 - \textit{Evaluating Sensitivity}: The angle of extinction ($\theta$) as determined from Gaussian fits of probability distributions (see methods) is plotted against the target concentration.

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**Figure B.2** - Evaluating Sensitivity: The angle of extinction ($\theta$) as determined from Gaussian fits of probability distributions (see methods) is plotted against the target concentration.
The flow cell design is schematically illustrated in Figure B.3A. Surfaces were prepared according to the procedures outlined in the methods. Upon observation of the LC orientation following ssDNA combing within the microfluidic cell, we did in fact see azimuthal LC orientation in the plane of the combing direction Figure B.3D-F. Figure B.3D shows a polarized micrograph at θ=0° of a LC cell created using a substrate prepared by stretching DNA within the microfluidic channel. The edges of the microfluidic channel were readily observed due to distortions in the LC alignment caused by the silicone rubber contact with the underlying GPTMS. It was clear that at θ=0°, extinction occurred in

Figure B.3: Stretching DNA in a microfluidic environment: (A) schematic illustration of the flow cell design (a=23.8; b = 49.0; c=34.0; d=2.0; e=7.0; f=radius=1.5 (mm)) (B) Brightfield image of the flow profile created as an air/water interface moves through the microfluidic environment (C) The relative height of the flow profile (y_m) is plotted against the channel position (x_ch) (D) A polarized light micrograph of a LC cell composed of a ssDNA functionalized substrate combed within a microfluidic channel and an opposing OTES SAM (E) The normalized intensity as determined from the polarized micrograph in (D) plotted against the cross sectional channel position (F) The probability of extinction at angle θ (p_ex,θ) plotted against θ.
the center of the channel. Furthermore, we used the quantitative analysis described in the methods and outlined in Figure B.3B,C,E,F to demonstrate that the probability for extinction at θ=0° is > 95%.

**B.4 Conclusions**

We have successfully shown that oligonucleotides induced specific azimuthal LC orientations that depend on DNA conformation. Future work in this area should focus on gaining a deeper understanding of the competition between the ssDNA and dsDNA anchoring strength at the interface toward developing a sensitive multiplexed device. In particular, the anchoring strength of purely ssDNA – vs - dsDNA modified substrates may clarify how much ssDNA can be tolerated when trying to identify low surface coverage of dsDNA. Furthermore, DNA hybridization should be detected within the microfluidic cell. Difficulties related to this goal have been encountered that were related to flow cell disassembly. When high quality GPTMS monolayers were used with the silicone rubber flow cell, the adhesive forces were too strong to disassemble the flow cell after use without damaging the substrate. However, when poor quality GPTMS monolayers were used the apparent coverage of dsDNA was too low to observe azimuthal alignment rotated 30° from the stretching direction. Thus, future work should involve designing a more robust flow cell, measuring the anchoring strengths associated with DNA alignment layers, and developing a detection scheme that enables multiplexed detection of DNA hybridization.

**B.5 References**


