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Liquid Crystal-based Imaging of Biomolecular Interactions at Roller Printed Protein Surfaces

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In this study, the orientational behavior of thermotropic liquid crystals (LC) supported on a film of protein receptors was examined. Avidin was roller printed and covalently immobilized onto the surface of gold using NHS/EDC chemistry. The orientation of nematic 4-cyano-4'-pentylbiphenyl (5CB) was found to be parallel to the plane of the printed avidin surface before incubation with a solution of biotin. However, protein-receptor complexation induced a random orientation of 5CB, where protein-receptor complexes disturbed the nanoscale topography of the printed protein surface. Atomic force microscopy and ellipsometry was used to confirm printing and the specific interaction of proteins. These results demonstrate that the combination of LC and roller printing can be used to detect specific interactions between biomolecules by manipulating the orientational behavior of LC to the printed protein surfaces.

Key Words: Liquid crystals, 4-Cyano-4'-pentylbiphenyl (5CB), Orientation of 5CB, Roller printing, Protein-receptor complexation

Introduction

Liquid crystalline materials hold great promise for use in a range of different sensing applications. The orientational properties of liquid crystals (LCs) enables the amplification and the transduction of biologically relevant binding events at nanostructured surfaces into optical outputs.[1-3] They can be used to image receptor-mediated binding of proteins at surfaces with submicrometer spatial resolution.[4,5] This method is potentially useful because it does not require the use of enzymatic or fluorescent labels, which largely complicate surface-based assays and prevent high levels of multiplexing.[6,7] In addition, this approach does not require the use of complex instrumentation[8] or laborious techniques.[9] One approach for using LC to sense biomolecular interactions on surfaces has exploited the nanometer-scale structure of self-assembled monolayers (SAMs) on obliquely deposited films of gold.[10-13] Although SAMs made from organosulfur compounds on Au surfaces permit a high level of control over the nanostructured surface, this approach requires the use of an ultrahigh-vacuum chamber for deposition of gold films.[14-16] Therefore, widespread use of this approach is highly limited.

In this study, a simple and versatile technique that permits the preparation of substrates for use in liquid crystal-based biomolecular assays and does not require use of complex instrumentation was developed. Our approach is based on the roller-printing of proteins by using a cylindrical stamp, which results in the formation of films of protein covalently attached to pre-activated surfaces on glass microscope slides. A schematic describing the procedure for roller printing and fabrication of optical cells using LC is shown in Figure 1.

The anisotropic surface structure created through this procedure possesses the following essential properties that enabled us to make LC-based biomolecular assays. First, roller printed films of protein can result in a uniform orientation of the liquid crystals. Second, the printed proteins can specifically bind to targeted molecules in solution. Third, it is easy to remove proteins that have nonspecifically bound to the printed surface by mild rinsing. Fourth, the binding of the targeted molecules to the printed protein films induces measurable changes in the alignment of LC.

In this study, we demonstrated the feasibility of our approach by roller printing and covalently immobilizing avidin onto the pre-activated surface of gold. The orientation of nematic 4-cyano-4'-pentylbiphenyl (5CB) was found to be parallel to the plane of the printed avidin surface before incubation with a solution of biotin. However, protein-receptor complexation induced a random orientation of 5CB, where protein-receptor complexes disturbed the nanoscale topography of the printed protein surface. Atomic force microscopy (AFM) and ellipsometry was used to characterize the structure of the roller printed films of avidin before and after binding of biotin.

Experimental Details

Materials. Titanium (99.999%) and gold (99.999%) were obtained from International Advanced Materials (NewYork, NY). The glass microscope slides were Fisher's Finest, premium grade slides obtained from Fisher Scientific (Pittsbur-

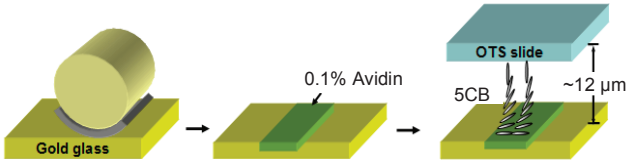

Figure 1. Schematic illustration of the procedure used for roller printing and imaging of proteins using liquid crystals.

gh, PA). The nematic liquid crystal 4-cyano-4'-pentylbiphenyl (5CB), manufactured by BDH, was purchased from EM Industries (Hawthorne, NY). The polished silicon (100) wafers were from Silicon Sense (Nashua, NH). The PDMS stamps were prepared from Sylgard 184 (Dow Corning, Midland, MI). Octyltrichlorosilane (OTS), 3-aminopropyltriethoxysilane (APES), succinic anhydride (SA), 11-mercaptoundecanoic acid, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), avidin, biotin, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. All protein solutions were made from PBS, pH 7.4. All aqueous solutions were prepared with high purity deionized water (18 MΩ cm) using a Milli-Q water purification system (Millipore, Bedford, MA).

Cleaning of substrates. The glass microscope slides were cleaned using a "piranha solution" (70% H_2SO_4/30% H_2O_2, *Caution: "piranha solution" reacts violently with organic materials and should be handled with extreme caution; do not store the solution in closed containers*) for 1 h at 80 °C. After removal from the cleaning solution, the substrates were rinsed with copious amounts of deionized water, ethanol, and methanol and dried under a stream of gaseous N_2. The cleaned substrates were stored overnight in an oven at 120 °C.

Preparation of octyltrichlorosilane (OTS)-treated glass slides. The piranha-cleaned glass slides were immersed into an OTS/*n*-heptane solution for 30 min. The slides were rinsed with methylene chloride and dried under a stream of N_2. The OTS slides were tested for homeotropic alignment by observing the orientation of 5CB sandwiched between two OTS slides. Any slide not displaying homeotropic alignment was discarded.

Deposition of gold films. For use in combination with liquid crystals, semitransparent films of gold with thicknesses of 200 Å were deposited onto clean glass microscope slides mounted on rotating planetaries using an electron beam evaporator. The rotation of the substrates on the planetary ensured that the gold was deposited without a preferred direction of incidence. A layer of titanium (thickness of 80 Å) was used to promote adhesion between the glass microscope slide and the film of gold. The rates of deposition of gold and titanium were 0.2 Å/s. The pressure in the evaporator was $< 5 \times 10^{-7}$ Torr during deposition.

Preparation of carboxylic acid-terminated self-assembled monolayer. Self-assembled monolayers were formed on the surfaces of gold films by immersion of the films in ethanolic solutions containing 2 mM 11-mercaptoundecanoic acid ($HOOC(CH_2)_{10}SH$). After 2 h of immersion, the slides were rinsed with ethanol and dried under a stream of gaseous N_2. The surface was activated with NHS ester right before the proteins were printed.

Preparation of stamps. PDMS stamps were prepared by casting Sylgard 184 on a silicon master placed in a Petri dish and cured overnight at 80 °C. The master was silanized with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane vapor (overnight under vacuum) to aid in the release of the PDMS. The PDMS was cut into 15 mm × 3 mm stamps for the next step.

Roller printing of proteins. The cylindrical stamp was inked with antibody by covering the entire stamp with 1 mg/mL avidin in phosphate buffer saline (PBS) for 30 min. The inked stamp was briefly rinsed with water, dried under a stream of gaseous N_2, and then wrapped around a glass cylinder using double-sided tape. The avidin layer was then printed by rolling the stamp onto the surface containing a carboxylate-terminated monolayer that was pre-activated with NHS ester. During rolling, pressure was gently applied to ensure contact between the stamp and the monolayer was maintained.

Binding of proteins form solution. Specific and nonspecific binding of proteins to the roller printed films of covalently immobilized avidin were performed by incubation of the substrates in PBS solutions of proteins (pH 7.4) for 1 h at 35 °C. Solutions of 1 mg/mL of biotin and bovine serum albumin (BSA) in 10 mM PBS were used. The substrates were sequentially rinsed with 0.01% Triton X-100 in PBS, PBS, and deionized water. The substrates were dried under a stream of N_2 upon removal from the protein solutions.

Preparation of optical cells. Optical cells were prepared by pairing a surface roller printed with avidin with an OTS-treated glass microscope slide. The slides were aligned facing each other, and kept apart by inserting a film spacer (thickness of ~12 μm) at the edge of the surfaces. The cells were held together using mini binder clips. The cells were heated to ~40 °C by placing on a warm plate and using a heat gun. The 5CB, heated up to its isotropic phase (> 35 °C) within a glass syringe, was dispensed onto the edge of the optical cells. The 5CB was then drawn into the space between the two surfaces by capillary forces. The cell was slowly cooled to room temperature over a period of 1 h. Upon cooling, the 5CB transitioned from the isotropic to the nematic phase.

Image capture. A polarized light microscope (ECLIPSE LV 100POL, Nikon, Tokyo, Japan) was used to image the optical textures formed by light transmitted through the optical cells filled with nematic 5CB. All images were obtained using a 4× objective lens between crossed polarizers. Images of the optical appearance of each liquid crystal cell were captured with a digital camera (DS-2Mv, Nikon, Tokyo, Japan) that was attached to the polarized light microscope. The images were captured at a resolution of 1600 × 1200 pixels, a gain of 1.00×, and a shutter speed of 1/10 s.

Ellipsometry. Ellipsometric thicknesses were measured using an Elli-SE(UV)-FM8 (Ellipsotechnology) at a wavelength of 380 ~ 1100 nm and an angle of incidence of 70°. All measurements were performed using silicon wafers that were treated with the same procedure used to prepare the roller printed films of avidin on the glass microscope slides. The increase in optical thickness of the organic layer after binding of proteins was calculated using a simple two-layer model (organic layer/effective substrate of SiO_2/Si) by assuming the refractive index of the organic layers was 1.18.

Atomic force microscopy. Images of the roller printed films of avidin and bound proteins were obtained by AFM using a Veeco Nanoscope IIIa instrument (Santa Barbara, CA) operating in tapping mode. Samples were imaged under ambient conditions using silicon tips with an average radius of ~10 nm. Images were acquired at a scan rate of 1.0 Hz with 256 sample points per line.

Results and Discussion

Orientation of 5CB on roller printed films of covalently immobilized avidin. This study was conducted to test the hypothesis that roller printed proteins could be imaged with liquid crystals by designing surfaces that possess the four essential properties described in the introduction. To determine the orientational properties of nematic 5CB on these surfaces, an optical cell containing two surfaces separated by a thin film spacer was assembled. One surface consisted of a carboxylate-terminated monolayer formed from 11-mercaptoundecanoic acid on a flat film of gold deposited on a glass microscope slide. The other surface was a glass microscope slide functionalized with octyltrichlorosilane (OTS). OTS is known to cause homeotropic (perpendicular) orientation of 5CB.[17,18] The thickness of the LC film that was deposited into the cavity between the two surfaces was measured to be 12 ~ 13 μm. The orientation of 5CB within the optical cell was determined by rotating the optical cell between crossed polars on a microscope stage. The orientation of the sample on the microscope stage was defined as the angle between the direction of protein printing and the polarizer of the optical microscope.

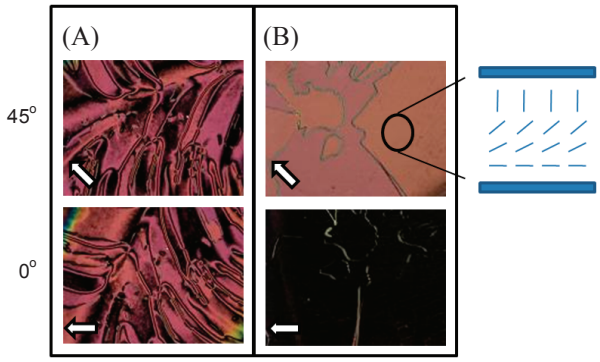

Figure 2. Optical appearance (crossed polarizers) of nematic 5CB supported on carboxylic acid-terminated monolayers (A) before and (B) after contact with a cylindrical stamp that had been incubated in 1 mg/mL avidin. The lower images shown in (A) and (B) were obtained after rotation of the cell shown in the above images by 45°. The white arrows indicate the direction of roller printing and the horizontal dimension of each image was 3.2 mm.

Figure 2A shows the optical appearance of a film of nematic 5CB supported on the carboxylic acid-terminated monolayers before and after contact with the cylindrical stamp that had been incubated in 1 mg/mL avidin. When avidin was not printed on this surface, the optical appearance of the liquid crystals was complex and nonuniform (Figure 2A). The nonuniform appearance of the LC indicated that the nematic phase of 5CB was anchored on this monolayer film without a preferred azimuthal orientation. In contrast, when 5CB was supported on the roller printed films of avidin, the optical appearance of 5CB was uniform and featureless (Figure 2B). This result indicates that the liquid crystals were uniformly oriented on the surface of the roller printed film of avidin. By using a quarter wave plate, the liquid crystals of 5CB were shown to be aligned on these surfaces in a direction that was parallel to the direction of roller printing. When rotated between crossed polarizers, the optical appearance of 5CB anchored between the roller printed films of avidin was found to modulate between dark and bright (Figure 2B). The liquid crystals appeared dark when the optical axis of the nematic phase was aligned with either the polarizer or the analyzer. Based on these results, we concluded that the roller printed films of chemically immobilized avidin induced a "uniform" and "planar" anchoring of nematic phases of 5CB in a direction that was parallel to the direction of printing.

Orientation of 5CB after specific binding of biotin to roller printed films of avidin. The specific binding of a protein to the roller printed film of avidin and the consequence of binding on the orientations of LC was then examined. Figure 3A shows

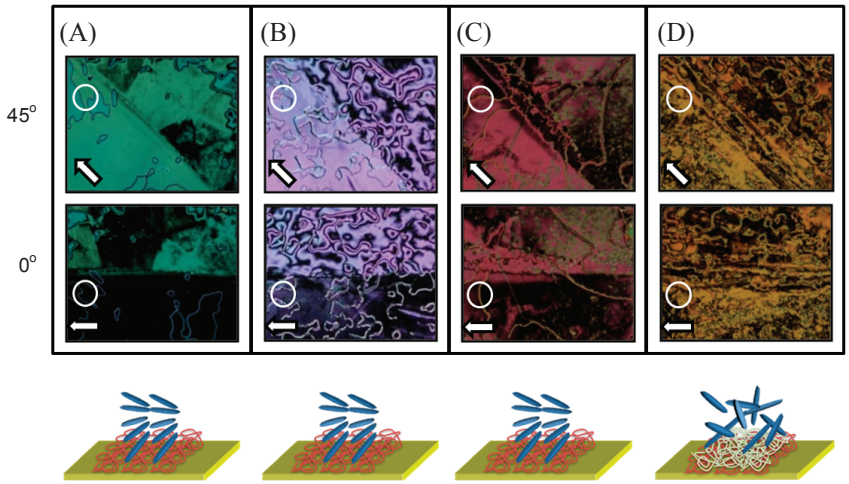

Figure 3. (A) Optical image (crossed polarizers) of a film of nematic 5CB in contact with a carboxylic acid-terminated surface roller printed with a stamp that had been incubated with a solution of avidin. The roller printed surfaces were immersed in a solution of (B) aqueous PBS (C) aqueous PBS containing 1 mg/mL BSA, and (D) aqueous PBS containing 1 mg/mL biotin at 35 °C for 1 h. Following the withdrawal of the surface from each solution, the surface was sequentially rinsed with 0.01% Triton X-100 in PBS, PBS, and water, and then dried under a stream of N_2 before contact with 5CB. The lower images were obtained after rotation of the cell shown in the above images by 45°. The white arrows indicate the direction of roller printing and the horizontal dimension of each image was 3.2 mm.

the optical image (crossed polarizers) of a film of nematic 5CB in contact with a carboxylate-terminated surface roller printed with a stamp that had been incubated with a solution avidin. The area that appears nonuniform corresponds to the regions of the carboxylate-terminated surface that were not contacted by the cylindrical stamp. In contrast, the LC appeared bright green in the regions that had contacted the stamp incubated with avidin. Rotation of the sample between crossed polars revealed a parallel orientation of the LC in this region, which had an azimuthal orientation that was parallel to the direction of roller printing.

We next investigated the effects of specific binding of proteins to the printed avidin surface on the orientations of the liquid crystals. A printed film of covalently immobilized avidin was incubated with a solution of aqueous PBS containing 1 mg/mL biotin at 35 °C for 1 h. Following removal of the surface from the biotin solution, the surface was rinsed sequentially with 0.01% Triton X-100 in PBS, PBS, and water and then dried under a stream of N_2. Figure 3D shows an optical image of a film of 5CB that was in contact with this surface. Under these conditions, the optical appearance of LC was highly nonuniform. Rotation of the sample between crossed polarizers did not result in reproducible modulation in the intensity of light transmitted through the sample. This result suggests that specific binding of biotin erased the anisotropic structure of the roller printed film of avidin, which was responsible for the uniform alignment of LC.

Although the above results are promising, we suspected that the level of nonspecific adsorption of proteins on the printed protein surfaces would be high enough that the uniform orientation of LC would be erased by the adsorbed proteins. Nonspecific adsorption of proteins on these surfaces will prevent their use as substrates for biomolecular assays that are based on LC, because the nonspecific adsorption will mask the effects of specific binding of proteins to the surfaces. To test this possibility, we conducted two control experiments; incubation of the avidin surface with an aqueous PBS solution and incubation of the avidin surface with PBS containing 1 mg/mL BSA at 35 °C for 1 h. Following removal of the surface from each solution, the stamp was sequentially rinsed with 0.01% Triton X-100 in PBS, PBS, and water and then dried under a stream of N_2. Figure 3B and C show the optical appearance of 5CB anchored on a printed film of avidin that was immersed and withdrawn from the aqueous PBS and PBS containing 1 mg/mL BSA, respectively. Although defects were evident in the optical appearance of the LC supported on the film immersed into the control solutions, the bulk of the LCs remained uniformly oriented. Therefore, we concluded that the level of nonspecific adsorption of BSA on the roller printed film of avidin after sequential rinsing was insufficient to disrupt the uniform anchoring of the LC. This result is in stark contrasts with those obtained for the specific binding of biotin to the printed film of avidin (Figure 3D), where the specific protein interactions triggered the nonuniform anchoring of LC.

Ellipsometric measurement and topographical analysis of protein binding to roller printed films of avidin. To determine whether the orientational transition of nematic 5CB was due to a change in the amount of protein binding, ellipsometry was used to measure the extent of protein binding to the roller printed films of avidin. A silicon wafer was first functionalized with carboxylic acid using 3-aminopropyltriethoxysilane (APES) and succinic anhydride (SA). Avidin was then roller printed and covalently immobilized onto this surface using NHS/EDC chemistry.

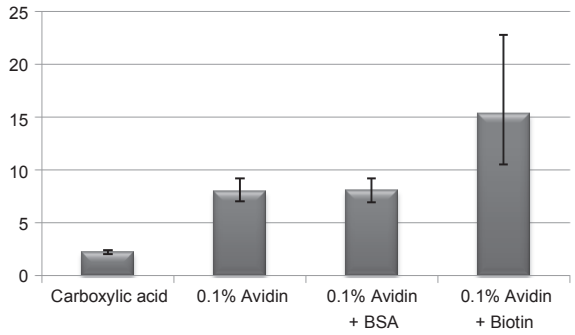

Figure 4. Ellipsometric thicknesses of proteins adsorbed onto the roller printed films of avidin.

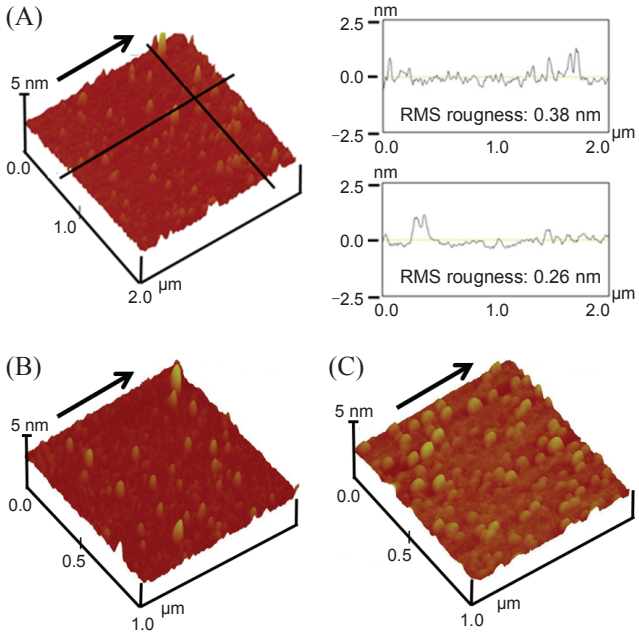

Figure 5. AFM images of roller printed films of avidin (A) prior to incubation, (B) after incubation with aqueous PBS containing 1 mg/mL BSA, and (C) after incubation with aqueous PBS containing 1 mg/mL biotin. The black arrows in each image indicate the shear direction, and the two profiles in image (A) correspond to the cross-sectional views.

As shown in Figure 4, the ellipsometric thickness did not measurably change due to nonspecific protein adsorption. In contract, specific binding of biotin to the printed avidin surface resulted in a significant increase of optical thickness. The ellipsometric thickness of the bound biotin was determined to be 7.3 nm. Given that the size of the biotin molecule used in our study was approximately ~5 nm (peroxidase-biotinamidcoproyl conjugate from SIGMA: The molecular weight of horseradish

peroxidase is approx 44 kDa. The degree of substitution is 2.2 moles of biotin/mole), this increase in elliposmetric thickness (7.3 nm) approximately corresponds to monolayer coverage.

We also measured the change in the nanometer-scale topography of films of roller printed avidin before and after incubation with protein solutions. Figure 5A shows a topographical image and cross-sectional profiles of a film of avidin. The effect of roller printing on the topographical structure of these surfaces was observed. Inspection of cross-sectional lines revealed that roller printing introduced an anisotropic topography into the films of avidin. Incubation of the surface with a nonspecific protein (BSA) solution induced no significant changes in topography (Figure 5B). In contrast, the immersion of the avidin surface into an aqueous solution of biotin resulted in a significant increase in the topography and thickness of the surface due to specific protein binding (Figure 5C). Comparison of Figures 5A and 5C clearly shows that the binding of biotin to the roller printed avidin masks the anisotropic topography measured by AFM in the absence of bound biotin. Based on these results, we concluded that the elimination of the anisotropic structure of the roller printed avidin surface due to the binding of biotin was responsible for the orientational transition of LC (from uniform planar to random) on theses surfaces.

Conclusion

The results of this study clearly demonstrate that roller printed films of avidin covalently immobilized on the surface of glass microscope slides can uniformly orient LC. Proteins that nonspecifically adsorbed from aqueous solutions were easily removed by mild rinsing to levels that were low enough to maintain uniform alignment of LC. In contrast, specific binding of proteins to these surfaces eliminated the anisotropic structure of the roller printed proteins and triggered an orientational transition of LC. These properties, when combined, suggest that roller printed films of proteins may be useful as substrates on which specific biomolecular interactions can be imaged by LC. Therefore, the results of this study establishes the generality of using roller printed films of proteins covalently immobilized on surfaces as substrates for protein chips that can be imaged by LC.

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