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Imaging the Enzymatic Reaction of Urease Using Liquid Crystal-Based pH Sensor

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In this study, real-time and label-free methods for monitoring the enzymatic reaction of urease, which releases ammonia through the hydrolysis of urea in an aqueous solution, were developed using a liquid crystal (LC)-based pH sensor. Nematic liquid crystal 4-cyano-4'-pentylbiphenyl (5CB), doped with 4'-pentyl-biphenyl-4-carboxylic acid (PBA), exhibited a shift in optical appearance from bright to dark when it was in contact with ammonia generated from the enzymatic reaction between urease and urea. This optical change was attributed to the anchoring transitions of LCs caused by hydrophobic interactions between the tails of deprotonted PBA (PBA^-) molecules and the LCs at the aqueous/LC interface. This novel technique holds great promise for the sensitive detection of urease along with its substrates and inhibitors.

Key Words : Liquid crystals, 4-cyano-4'-pentylbiphenyl (5CB), 4'-pentyl-biphenyl-4-carboxylic acid (PBA), Sensors, Urease

Introduction

The detection and analysis of enzymatic reactions important to various pathogens can help provide a better understanding of the pathology of disease and aid in the development of effective therapeutics.[1-3] Since traditional methods for detecting enzymes are usually discontinuous, time-consuming and require complex instrumentation or laborious techniques, innovative strategies for exploiting simple and portable biosensors with high sensitivity have been widely studied over the past decade.[4-9] The emerging liquid crystal (LC)-based sensor is one of the most promising techniques for high-throughput and label-free detection of enzymes in real-time.[10-12]

Due to its highly cooperative and long-range anchoring transitions, which propagate from aqueous/LC interfaces, LCs can be used to amplify and transduce molecular and biomolecular events into optical outputs visible by the naked eye.[13-15] Studies on highly sensitive LC-based biosensors based on the orientational transition of LCs involve pH indication,[16] enzymatic reactions,[16-18] DNA hybridation[19] and protein binding events.[20] In terms of pH sensors, amphiphiles containing pH-sensitive functional groups are usually employed to detect pH shifts related to ordering transitions of LCs. For example, Kinsinger *et al*. reported that the assembly of a synthetic polymer with pH-sensitive functional groups could be coupled to orientational transitions of LCs associated with pH shifts at aqueous/LC interfaces.[21] Optical responses of the LCs changed significantly when the pH of aqueous solutions shifted between 5 and 9, because of the rearrangement of polymers at the aqueous/LC interface. Bi *et al*. developed a new LC-based pH sensor to monitor small amounts of H^+ released from enzyamtic reactions in real-time, especially in an aqueous solution with a high buffer capacity.[16] 4'-pentyl-biphenyl-4-carboxylic acid (PBA), which contains a pH-functional group and has a similar structure to the nematic LC, 4-cyano-4'-pentylbiphenyl (5CB), was doped into 5CB. When a small quantity of H^+ was generated from the enzymatic hydrolysis of penicillin G in PBS (pH=7.0), the optical appearance of the acid-doped 5CB experienced a dark-to-bright shift, indicating an orientational transition of LCs, which was attributed to the protonation of PBA at the aqueous/LC interface.

Recently, we developed a LC-based sensor for the detection of urease and its inhibitors using UV-treated 5CB. Since the main product of the photochemical degradation of 5CB contains an acid group, the optical appearance of the LCs changed from bright to dark due to the enzymatic hydrolysis of urea, which can be decomposed into ammonia and carbon dioxide by urease. However, this was a relatively complex system and measuring the concentration of the carboxylic acid product in 5CB involved a relatively laborious process. Therefore, in this study, we developed an alternative system using LCs doped with a carboxylic acid surfactant for which the concentration was known. Due to the pH-sensitive property of PBA, we postulate that the orientational transitions of LCs could be obtained when ammonia was released from the reaction of urease and urea was in contact with the carboxylic acid-doped 5CB. Thus, this approach holds promise for monitoring the enzymatic activity of urease in the aqueous phase due to its rapid optical response. This ammonia-based LC sensor could be used as a new tool for real-time monitoring of urease and its inhibitors with high sensitivity and spatial resolution.

Experimental Details

Materials. Nematic liquid crystal 4-cyano-4'-pentylbiphenyl (5CB), manufactured by BDH, was purchased from EM industries (Hawthorne, NY). The premium glass microscope slides were obtained from Fisher Scientific (Pittsburgh, PA). Copper and gold specimen grids were purchased from

GILDER. Sulfuric acid, hydrogen peroxide (30% w/v), octyltrichlorosilane (OTS), 11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), 1-ethyl-2-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 4'-pentylbiphenyl-4-carboxylic acid (PBA), urease, urea were purchased from Sigma-Aldrich. All aqueous solutions were prepared with deionized water (18 MΩ cm), using a Milli-Q water purification system (Millipore, Bedford, MA).

Preparation of Urease-Modified Gold Grids. Gold grids 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*). Self-assembled monolayers (SAMs) were formed by immersing gold grids in 2 mM ethanolic solution of MUA for about 12 h, and then rinsed with copious ethanol and DI water. An aqueous solution of NHS/EDC (50 mM/200 mM) was used to activate the carboxylic aicd-terminated SAMs present at the surface. After 30 min, the gold grids were rinsed with copious water and immersed in a 2 μM urease solution for about 8 h at 4 °C in a refrigerator. Finally, the urease-modified grids were rinsed with DI water again and dried under nitrogen gas at room temperature.

Treatment of Glass Microscope Slides with OTS. Glass microscope slides were cleaned with "piranha solution" for 30 min at 80 °C. They were then rinsed with water, ethanol, and methanol, and dried under a stream of gaseous N_2, followed by heating to 120 °C overnight prior to OTS deposition. The "piranha-cleaned" glass slides were immersed into an OTS/n-heptane solution for 30 min. The slides were then rinsed with methylene chloride and dried under a stream of N_2.

Preparation of Optical Cells. The OTS-coated glass slides were fixed at the bottom of an eight-well chamber slide. TEM specimen grids were then placed onto the OTS-coated glass slide. 2.0 μL 0.4% PBA-doped 5CB (isotropic state) was dispensed into each grid and excess LC was removed by placing a 20 μL capillary tube in contact with the 5CB droplet on the grid. Subsequently, 500 μL aqueous solution of interest was immersed into the optical cell at room temperature.

Optical Examination of LC Textures. A polarized light microscope (ECLIPSE LV100POL, Nikon, Tokyo, Japan) was used to image the optical textures formed by polarized light transmitted through the optical cells filled with nematic 5CB. All images were obtained using a 4 × objective lens between crossed polarizers. The optical appearance of the LC was imaged using a digital camera (DS-2Mv, Nikon, Tokyo, Japan) 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.

Results and Discussion

Orientational Behaviors of the PBA-Doped 5CB Induced by the Combined Effect of pH and Electrolytes at Aqueous/LC Interfaces. The anchoring transitions in LCs can be coupled with various interfacial events, which can involve the amplification and transduction of chemical and biological interactions using liquid crystalline materials at the aqueous/LC interface. In this sensing system, the transducer is an approximately flat film obtained by depositing LCs in the holes of a transmission electron microscopy (TEM) grid with a thickness of 20 μm. The grid is supported on a glass slide modified with octyltrichlorosilane (OTS), which can trigger a perpendicular alignment of the LC molecules at the glass/LC interface. Prior to the addition of the aqueous phase, the 5CB layer appears dark through the polarized microscope due to the homeotropic alignment of LCs at the air/LC interface. After the LCs are immersed in the aqueous solution of interest, the optical appearance as imaged through crossed polarizers changes due to orientational changes in the anchored 5CB.[22]

In a previous study, PBA-doped 5CB was used to screen pH variations in sodium phosphate buffer (PBS).[18] A bright-to-dark change in the optical appearance, which indicated a planar-to-homeotropic transition of LCs, was observed under the polarized microscope when the pH of the PBS solution was increased from 6.0 to 7.0. This result suggested that the pH of the buffer influenced the self-assembly behavior of the deprotonated PBA (PBA^-) molecules and determined the orientation of LCs at the aqueous/LC interface. Considering the self-assembly behavior of PBA, we hypothesized that the mechanism behind the ordering orientations of LCs was the combined effect of the pH and electrolytes in the aqueous phase. Because electrolytes can screen electrostatic repulsions at the interface, the adsorption of the amphiphilic PBA^- molecules was promoted, which also contributed to the orientation of LCs.

When sodium hydroxide (pH=10) or 0.1 M NaCl dissolved in DI water was introduced into the optical cell, the optical appearance of the LCs was bright and colorful (Figs. 1A, B), indicating a planar orientation of the LCs at the aqueous/LC interface. In contrast, the LCs adopted a dark

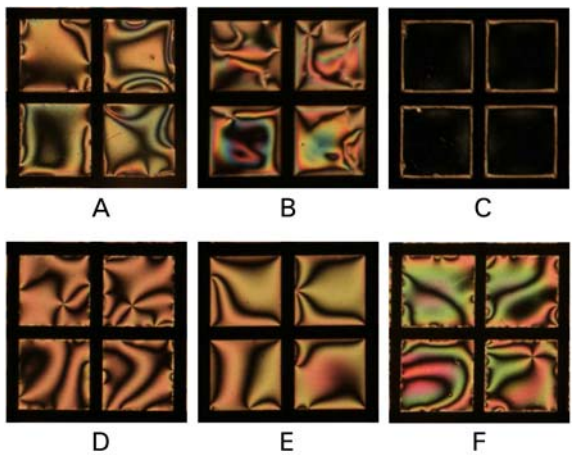


Figure 1. Polarized light microscopy images of the PBA-doped 5CB: (A) in contact of DI water (pH=10), (B) incubated in 0.1 M NaCl, (C) immersed in 0.1 M sodium chloride dissolved in DI water (pH=10). Optical images of pure 5CB: (D) in contact of DI water (pH=10), (E) incubated in 0.1 M NaCl, (F) immersed in 0.1 M sodium chloride dissolved in DI water (pH=10).

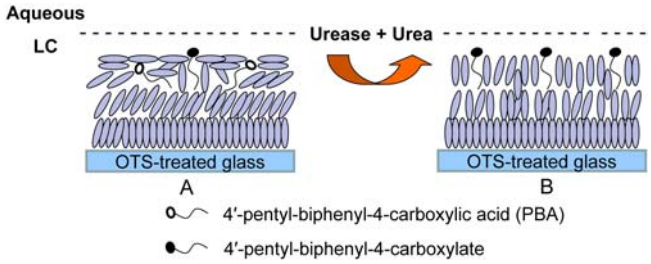
appearance when the PBA-doped 5CB was immersed in 0.1 M NaCl (pH=10) (Fig. 1C), which corresponds to a homeotropic alignment of LCs. When this experiment was repeated using pure 5CB, the LC layer remained bright and colorful (Figs. 1D-F). Thus, we concluded that the anchoring transitions of the PBA-doped 5CB were due to the combined effect of pH and electrolytes, which influences the self-assembly behavior of PBA^- molecules at the aqueous/LC interface and can affect the orientation transition and optical response of LCs.

Detection of the Enzymatic Reaction of Urease. Based on the combined effect of pH and electrolytes on the ordering of LCs at the aqueous/LC interface, we hypothesized that the orientation of LCs could be disturbed when the acid-doped 5CB was immersed with a mixture of urease and urea in an aqueous solution. Since urease hydrolyzes urea into ammonia and carbon dioxide, the pH of the aqueous solution should increase from an acidic to alkaline state through the ionization of the generated ammonia. Therefore, more PBA molecules would be inclined to deprotonate and assemble at the aqueous/LC interface, which changes the orientational ordering of LCs from a planar (Scheme 1A) to homeotropic state (Scheme 1B).

We pre-incubated an aqueous mixture of 100 nM urease and 0.5 M urea in DI water for 30 min at room temperature, and then transferred this solution onto the interface of the PBA-doped 5CB, which had been initially immersed in DI water. The optical appearance changed from bright (Fig. 2A) to dark (Fig. 2D) within 3 min, indicating an orientational transition of LCs from a planar to homeotropic state occurred at the aqueous/LC interface. The optical appearance of the LCs in the control experiments with 0.5 M urea or 100 nM urease did not change after incubation for 1 h (Figs. 2B, C), suggesting no anchoring transitions occurred in the orientation of the LCs. These results proved that PBA-doped 5CB could be used as an LC-based sensor for monitoring the enzymatic activity of urease in an aqueous solution.

A previous study showed that the orientational transition of LCs involving the adsorption of surfactants was caused by the hydrophobic interactions between the tails of the amphiphiles and the LCs at the aqueous/LC interface. The addition of salts could screen the electrostatic repulsions between the head groups of surfactants and facilitate the adsorption of the amphiphiles at the interface, thereby promoting an ordering transition in LCs. Since hydroxide and ammonium are produced in the aqueous phase through the ionization of ammonia due to the enzymatic reaction of urease and urea, a certain amount of PBA^- molecules can be generated and self-assemble at the aqueous/LC interface, which would induce orientational transitions of LCs. Besides the effect of hydroxide, the quaternary ammonium ions may also screen the electrostatic repulsions between the head groups of PBA^- molecules, which would also contribute to the formation of a dense PBA^- monolayer.

Detection Limit of the Enzyme Reaction. In order to test the detection limit of urease using the mixture-incubated method, the following experiment was conducted. 0.5 M urea and urease were mixed at different concentrations and preincubated for different times before being introduced into the optical cells. First, a mixture of 0.5 M urea and 100 nM urease was incubated for 3 min in advance, and then it was transferred onto the aqueous/LC interface. A completely bright to uniform black appearance of LCs was observed within 5 min (Fig. 3A), indicating that the LCs transitioned from a planar to homeotropic state. Next, an aqueous mixture of urea and 50 nM urease, also preincubated for



Scheme 1. The orientational alignment of the PBA-doped 5CB after the addition of the aqueous mixture of urease and urea into the optical cell: (A) planar orientation, (B) homeotropic orientation.

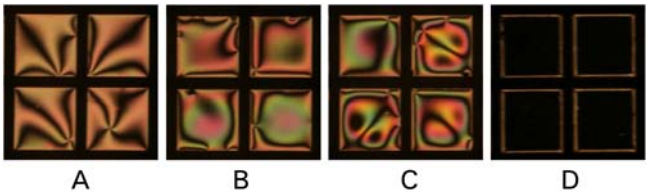


Figure 2. Optical images of the PBA-doped 5CB, which were acquired using a polarized light microscope: (A) in contact with DI water, (B) incubated with 0.5 M urea, (C) in contact with 100 nM urease, (D) immersed in a mixture of 0.5 M urea and 100 nM urease.

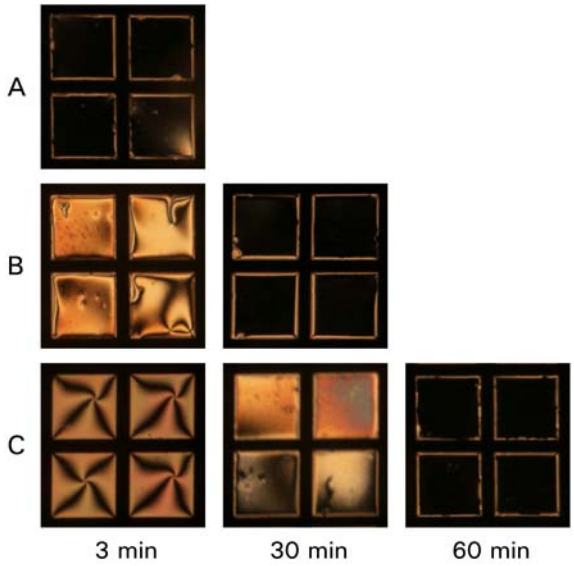


Figure 3. Optical images of the PBA-doped 5CB, which were acquired using a polarized light microscope: (A) immersed in 100 nM urease and 0.5 M urea, (B) incubated with 50 nM urease and 0.5 M urea, (C) in contact with 10 nM urease and 0.5 M urea. The time at the bottom represents the incubation time of the urease-urea mixtures prior to addition to the optical cells.

3 min, was immersed with the PBA-doped 5CB. Under these conditions, the color of the LCs shifted from a red and purple to orange and yellow color (Fig. 3B left), which suggests a planar-to-tilted alignment (relative to the surface normal) of the LCs. When the preincubation time was increased to 30 min, an orientational transition of LCs from a planar to homeotropic state was observed within 5 min (Fig. 3B right). A mixture of urea and 10 nM urease was also placed in contact with the acid-doped 5CB. When the preincubation time was 3 min, no obvious changes in the optical appearance were observed (Fig. 3C left), indicating that no orientational transitions occurred in the LCs. After preincubating the mixture for 30 min, the color of the LCs changed from red and purple to orange, yellow, and grey (Fig. 3C middle), which suggests a planar-to-tilted transition of the LCs. When the preincubation time was increased to 60 min, an orientational transition from planar to homeotropic state was observed (Fig. 3C right), which resulted in a bright-to-dark shift in the optical appearance of the LCs. These results suggest that the detection limit of this urease-sensing system was about 10 nM. However, lower concentrations of urease may be detected if the preincubation time of the mixture was extended further.

Enzymatic Hydrolysis of Urea Catalyzed by Urease Immobilized on Gold Grids. A previous study reported that enzymatic reactions could be sensitively monitored in real-time by immobilizing the enzyme on a TEM grid. During the hydrolysis of substrates, the orientation of the LCs was disturbed at the aqueous/LC interface, which influenced the optical appearance.[16] Since the PBA-doped 5CB showed an orientational transition when an aqueous mixture of urease and urea was transferred into the optical cell, we hypothesized that changes in optical appearance might also be observed when the urease-modified gold grid filled with the acid-doped 5CB was immersed into an aqueous solution containing urea. In order to prepare the urease-decorated grid, 11-mercaptoundecanoic acid (MUA) was used to modify the gold surface. After activating the carboxylic acid with NHS/EDC, urease was chemically immobilized on the gold grid. A bright and colorful image was obtained after the PBA-doped 5CB confined in the urease-modified gold grid was immersed with DI water (Fig. 4A), which suggests the LCs were in a planar orientation. Next, we replaced DI water with a 0.5 M urea solution. The optical image gradually changed from a red and green to grey (Fig. 4B) and black color (Fig. 4C), which corresponds to an orientational transition of LCs from a planar to tilted and perpendicular state. However, the optical image gradually reverted from uniform a dark to bright state within 15 min (Figs. 4D-F), implying an ordering transitions of LCs from the homeotropic to planar state.

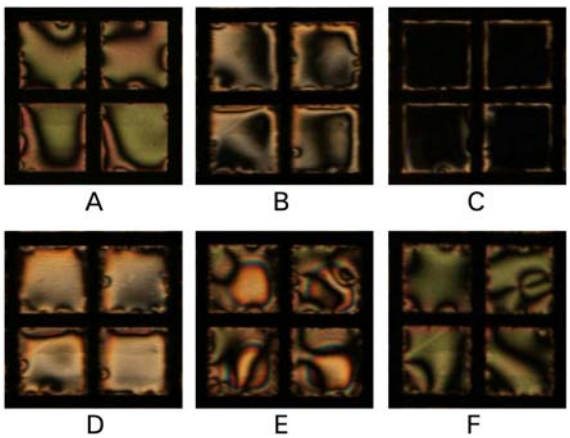


Figure 4. Polarized light microscopy images of the PBA-doped LCs: (A) incubated with DI water; after the addition of urea: (B) 1 min, (C) 2 min, (D) 4 min, (E) 8 min, (F) 15 min.

The mechanism behind these optical changes can be explained as follows: when DI water was added into the optical cell, the PBA^- was not sufficiently absorbed at the aqueous/LC interface, which could not disturb the planar orientation of LCs. Therefore, a bright image was observed. On the other hand, a bright-to-dark shift in the optical response of the LCs was obtained after replacing DI water with an aqueous solution of urea, because the ammonia generated from the enzymatic reactions could locally influence the orientational behavior of 5CB. Due to the ionization of ammonia, more PBA^- molecules self-assembled at the aqueous/LC interface. When the areal density of the amphiphiles was sufficient enough to induce an orientational transition of the LCs, the optical appearance changed from a bright to dark state. Although the PBA^- molecules formed a dense monolayer at the interface, they could also diffuse into the bulk solution at the same time. When the adsorption of the PBA^- molecules could not compensate for the diffusion of the amphiphiles, the areal density of the PBA^- molecules at the interface sustaining the homeotropic orientation of LCs began to decrease at the interface, and the orientation of LCs returned to a planar state, resulting in a change in the optical appearance from dark to bright.

Conclusion

In summary, we investigated the combined effect of pH and electrolytes on the orientational behaviors of the PBA-doped 5CB at the aqueous/LC interface and demonstrated the feasibility of monitoring the enzymatic activity of urease using 5CB doped with a carboxylic acid surfactant. The optical appearance of the LCs changed from bright to dark state after introducing a mixture of urease and urea into the optical cell. The same optical response was also observed when the PBA-doped 5CB confined in the urease-decorated grid was immersed in an aqueous urea solution. The hydrophobic interactions between the tails of the PBA^- molecules and the LCs were responsible for the orientational transition of LCs. These results showed that the PBA-doped 5CB may be used to monitor enzyme reactions that are sensitive to pH change.

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References and Notes

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