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Real-Time Liquid Crystal pH Sensor for Monitoring Enzymatic Activities of Penicillinase

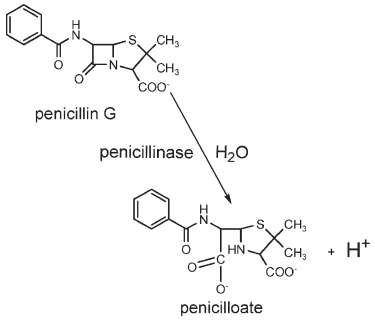
By *Xinyan Bi, Deny Hartono,* and *Kun-Lin Yang**

A liquid crystal (LC)-based pH sensor for real-time monitoring of changes in localized pH values near a solid surface is reported, along with its application for the detection of enzymatic activities. It is found that 4-cyano-4′-pentylbiphenyl (5CB), when doped with 4′-pentyl-biphenyl-4-carboxylic acid (PBA), shows a bright-to-dark optical response to a very small change in pH (from 6.9 to 7.0). The pH-driven optical response can be explained by using orientational transitions of 5CB induced by the protonation and deprotonation of PBA at the aqueous/LC interface. Because of its high pH sensitivity, the LC-based sensor is further exploited for monitoring local pH changes resulting from enzymatic reactions. As a proof of concept, the hydrolysis of penicillin G by surface-immobilized penicillinase is monitored using the system, even when the concentration of penicillin G is as low as 1 nM. This type of LC-based sensor may find potential utilities in high-throughput screening of enzyme substrates and enzyme inhibitors.

1. Introduction

Monitoring pH values is important in many chemical and biological processes, clinical analyses, environmental science, and oceanography. Although pH sensors made of glass electrodes are readily available for monitoring pH values in aqueous solutions, they are not suitable for measuring pH in solutions with small volumes. Recently, many novel techniques, including fluorescence[1] and pH-sensitive field-effect transistors (pHFETs),[2] have been developed to address this issue. Nevertheless, the pH measured using pHFETs is an average value over the entire sensor surface. Thus, it is often difficult to probe changes in localized pH values caused by surface reactions. Herein, we sought to exploit the optical properties of LCs and design a label-free pH sensor with good spatial resolution for monitoring changes in localized pH values resulting from enzymatic activities.

Penicillin G, which is a group of β-lactam antibiotics, has been widely used for treating bacterial infection, such as pneumococcal pneumonia.[3] However, almost as soon as penicillin was introduced, penicillin-resistant bacteria began to appear.[4] These bacteria produce the enzyme penicillinase, which is able to catalyze the hydrolysis of penicillin G as shown in Equation 1. After penicillin G is hydrolyzed, H^+ is released and the pH value is decreased as a result.[5,6]



(1)

Therefore, detecting penicillinase is possible by using pH changes associated with this reaction. Recently, some pioneering studies by Abbott's group have successfully demonstrated the utility of LCs to transduce and amplify molecular events at an aqueous/LC interface into optical images visible with the naked eye.[7] The LC-based sensor is sufficiently simple, label-free, and provides good spatial resolution.[8] Because anchoring of LCs at the aqueous/LC interface is controlled by energy at the scale of 10^{-2} to10^{-3} mJ m^{-2}, it is possible to couple the orientations of LCs to the presence of surfactants,[9] lipids,[10,11] proteins,[10] and synthetic polymers[12] adsorbed at the aqueous/LC interface. Moreover, when these surfactants or polymers contain pH-sensitive functional groups, orientations of LCs become sensitive to pH changes in the aqueous phase. For example, Kinsinger et al. designed a polymer-functionalized aqueous/LC interface (by conjugation of poly(ethylene imine) with *N*-[3-(dimethylamino)-propyl]acrylamide) to obtain an LC sensor that responded reversibly to pH changes in the aqueous phase.[12] They demonstrated that the pH-dependent changes in the orientation and optical appearance of LCs can be attributed to changes in the ordering of the polymer at the interface. However, they only observed different optical appearances of the LCs at pH = 9.0 and 5.0. Thus, whether this system is suitable for detecting very small pH changes is still unclear. Moreover, the response time of this system is very long (10 h).

To address the need for detecting small pH changes and the issue of slow response time reported previously, we designed a new LC-based pH sensor by doping 4-cyano-4′-pentylbiphenyl (5CB) with 4′-pentyl-biphenyl-4-carboxylic acid (PBA), which has a pH-sensitive functional group and a similar molecular structure to 5CB. We also studied the feasibility of using the

[*] Prof. K.-L. Yang, X. Bi, D. Hartono
Department of Chemical and Biomolecular Engineering
National University of Singapore
4 Engineering Drive 4, 117576 (Singapore)
E-mail: cheyk@nus.edu.sg

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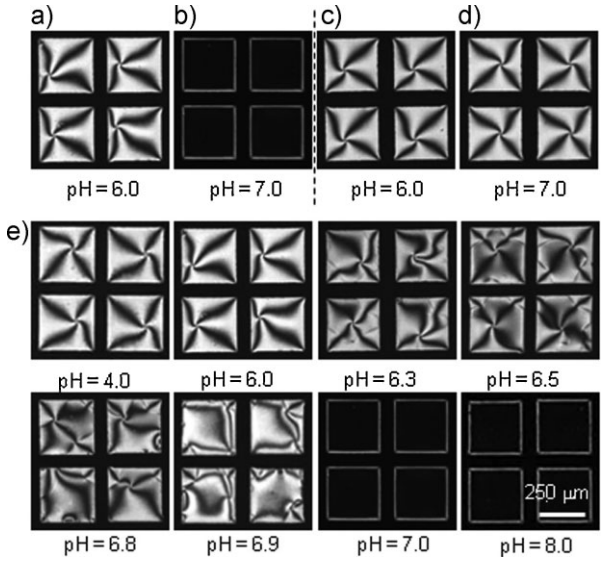


Figure 1. Effect of pH on the optical images of a,b) 5CB doped with 0.3% of PBA, c,d) pure 5CB, and e) 5CB doped with 0.3% of PBA.

LC-based pH sensor for monitoring H^+ released from enzymatic reactions in real time. The main challenge for this system is how to detect a small amount of H^+ released from an enzymatic reaction, especially in a solution with high buffer capacity. Nevertheless, we hypothesize that the release of H^+ still can lead to localized and temporal pH changes, which can be detected by a highly sensitive pH sensor with good spatial resolution.

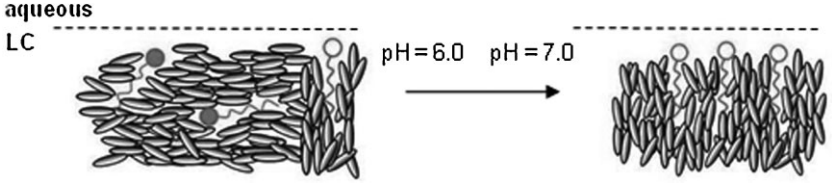
2. Results and Discussion

2.1. Orientational Response of Carboxylic-Acid-Doped 5CB to pH

Our first goal was to develop an LC-based pH sensor that gives fast response to small pH changes. Since 5CB does not contain any pH-sensitive functional groups, we doped it with 0.3% of PBA. We selected PBA because 1) it has a carboxylic acid moiety, which is sensitive to pH in aqueous solutions, and 2) its amphiphilic property may cause the molecule to self-assemble at the aqueous/LC interface, similar to the behavior of surfactants in the aqueous/LC system reported earlier.[9] 3) Additionally, the molecular structure of PBA is similar to 5CB; therefore, it has a better chance to interact with 5CB and influence the orientations of 5CB more effectively. The doped 5CB was then confined in a transmission electron microscopy (TEM) grid—which prevents the dewetting of 5CB—supported on a *N,N*-dimethyl-*n*-octadecyl-3-aminopropyltrimethoxysilyl chloride (DMOAP)-coated glass.[9a] When the whole system was immersed in sodium phosphate buffer (pH = 6.0), a bright image was observed under crossed polarizers (Fig. 1a). Interestingly, when we increased the pH of the sodium phosphate buffer to 7.0, the LC image became dark immediately (Fig. 1b). In contrast, when undoped 5CB was used in this experiment, 5CB appeared bright at pH = 6.0 and 7.0 (Fig. 1c,d). From these results, we can conclude that the optical image of 5CB, when doped with PBA, becomes very sensitive to changes in pH between 6.0 and 7.0. It is also obvious that PBA plays an important role in the response of 5CB to pH.

It is well known that bright images of LCs are caused by planar orientations of LCs at the aqueous/LC interface, while dark images of LCs are caused by homeotropic orientations. Moreover, past studies have shown that adsorption of surfactants at the aqueous/LC interface often leads to a planar-to-homeotropic transition in the orientation of the LCs when the density of a surfactant exceeds a critical value. In our system, since a similar transition occurs between pH = 6.0 and 7.0, we can postulate that the density of deprotonated PBA increases with the pH value until it exceeds a critical value at pH = 7.0 (Scheme 1) and that causes the bright-to-dark optical response observed in Figure 1a and b. To investigate the response to pH further, we immersed the PBA-doped 5CB in buffer solutions with pH values ranging from 4.0 to 8.0. Our experimental results in Figure 1e show that a bright-to-dark transition is triggered by a very small pH change (i.e., ≈0.1). The discontinuous transition between pH = 6.9 and 7.0 also implies that the LC pH sensor is an "all-or-nothing" type of sensor, which can offer many advantages that traditional pH sensors do not have.

Although it is apparent that PBA participates in the orientational response of 5CB to pH, the actual mechanism that leads to this phenomenon is still unclear. To shed light on the mechanism, we selected other molecules which also contain carboxylic acid groups and studied whether 5CB doped with these molecules also shows similar responses to changes in pH. First, we doped 5CB with 0.3% of 4-biphenylcarboxylic acid, which is similar to PBA; however, this molecule does not have a long hydrocarbon chain. After immersing 4-biphenylcarboxylic-acid-doped 5CB in aqueous solutions having different pH (from pH = 2.0 to 8.0), we observed that the images of the LCs were always bright regardless of the pH (Fig. 2a). Thus, we propose that the long hydrocarbon chain of PBA must play an important role in controlling the orientations of 5CB. Similarly, acetic-acid-doped 5CB also showed no response to changes in pH from 2.0 to 8.0 (Fig. 2b). This is consistent with the proposition that a long hydrocarbon chain in the dopant is needed for coupling the orientational behavior of 5CB to pH. To test this proposition, we doped 5CB with 0.3% of lauric acid, which has a carboxylic acid group and a long hydrocarbon chain. Figure 2c shows that the lauric-acid-doped 5CB appears bright at pH = 2.0 and 4.0, but it becomes dark at pH = 5.0 and 8.0. Therefore, this result supports our proposition. We also note that the bright-to-dark transition point occurs around pH ≈4.0–5.0, which coincides with the pK_a of lauric acid (≈4.95).[13] Thus, the bright-to-dark transition can be attributed to the protonation/deprotonation of lauric acid. Because the deprotonated lauric acid



Scheme 1. Orientational transition of 5CB caused by increasing pH from 6.0 to 7.0 in the aqueous solution.

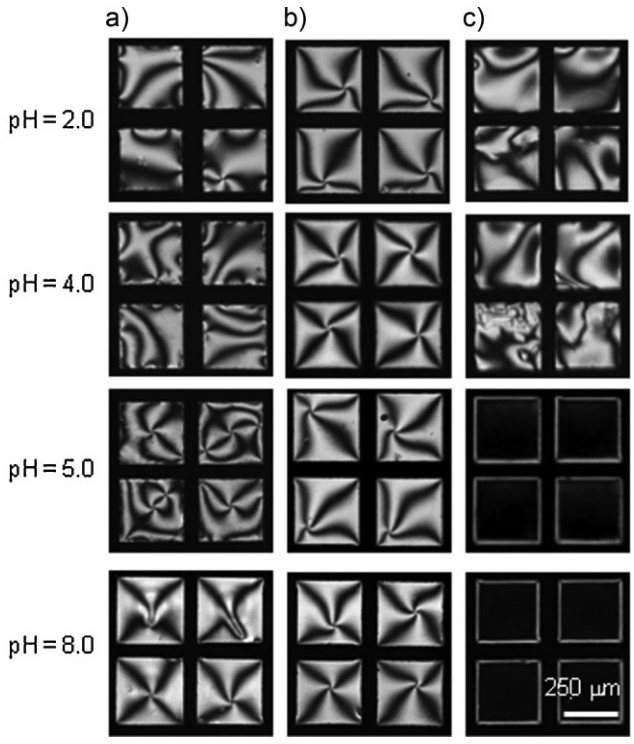


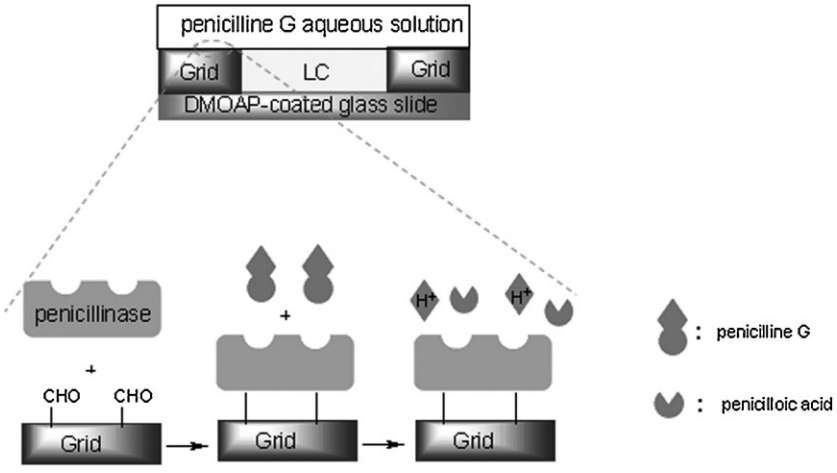
Figure 2. Optical images (crossed polars) of several acid-doped 5CBs and their optical responses to changes in pH. These acid dopants are a) 4-biphenylcarboxylic acid, b) acetic acid, and c) lauric acid.

is negatively charged, it is more amphiphilic than the protonated one. When the pH increases, the density of deprotonated lauric acid at the aqueous/LC interface increases, leading to the bright-to-dark transition in the appearance of 5CB.

However, the protonation/deprotonation theory may not be sufficient to account for the optical response in the PBA-doped 5CB as shown in Figure 1. Because the pK_a for PBA is 3.46, 99.96% of PBA is already in the deprotonated form at pH = 6.9. Increasing the pH value by 0.1 to pH = 7.0 will only cause another 0.01% of PBA to deprotonate, and that can only lead to a minimal increase in the density of deprotonated PBA at the aqueous/LC interface. Surprisingly, this small change leads to orientational response of 5CB and the bright-to-dark transition as shown in Figure 1. There are two possible explanations. First, the pK_a value for PBA at the interface is sufficiently different from that in the bulk. Because there is no reported pK_a value for PBA at the interface, we are unable to confirm this. However, we point out that lauric acid whose pK_a is similar to PBA does not exhibit this unusual phenomon. The transition point (pH ≈4.0 – 5.0) of lauric acid actually coincides with its pK_a in the bulk. The second explanation is the sensitivity of 5CB is extremely high. As pointed out by Abbott et al., an undetectable increase in the density of a ganglioside GM_1 can lead to a similar bright-to-dark transition in 5CB.[14]

2.2. Detecting H^+ Releasing from Enzymatic Reactions

Next, we sought to apply the LC-based pH sensor to develop a biosensor for detecting H^+ released from an enzymatic reaction. The advantages of the LC-based pH sensor include its unusually high sensitivity to pH and its ability to monitor localized pH values in a real-time manner. As a proof of concept, we selected the hydrolysis of penicillin G catalyzed by penicillinase as our model enzymatic reaction. As shown in Equation 1, when penicillin G molecules are hydrolyzed, some H^+ will be released. However, most H^+ will be neutralized by the buffer eventually. Thus, we hypothesized that pH changes associated with the enzymatic reaction is only temporal and highly localized (near the immobilized enzyme only). On the basis of this hypothesis, we designed an experimental system as shown in Scheme 2. First, we immobilized penicillinase on the metal bars of a copper grid. Then, the hollow square regions of the grid were filled with PBA-doped 5CB. Under this configuration, the PBA-doped 5CB was in the proximity of the immobilized penicillinase. Finally, the entire system was immersed into sodium phosphate buffer (pH = 7.0) containing penicillin G. Figure 3a shows that the optical image of the LCs becomes bright in the presence of penicillin G. For comparison, the same system was immersed in pure sodium phosphate buffer without penicillin G. Figure 3b shows that the LCs remains dark in this case. This result implies that penicillin G can be hydrolyzed by penicillinase immobilized on the copper grid, and the released H^+ can lower the local pH and cause the LCs to become bright. To confirm that the color change was indeed caused by the H^+ generated in the enzymatic reaction, we also immersed an unmodified copper grid (without penicillinase) in the penicillin G solution. Figure 3c shows that the image of the LCs remains dark, suggesting that penicillin G alone does not change the



Scheme 2. Configuration of an LC-based pH sensor is used to monitor highly localized pH changes resulting from enzymatic reactions. A copper grid coated with penicillinase is supported on a DMOAP-coated glass slide. A pH-sensitive LC, 5CB doped with PBA, is confined within the holes of the grid. The entire system is then immersed into a solution containing penicillin G. Zoom-in: schematic illustrations showing the immobilization of penicillinase and the enzymatic reaction of penicillinase on the surface of the copper grid.

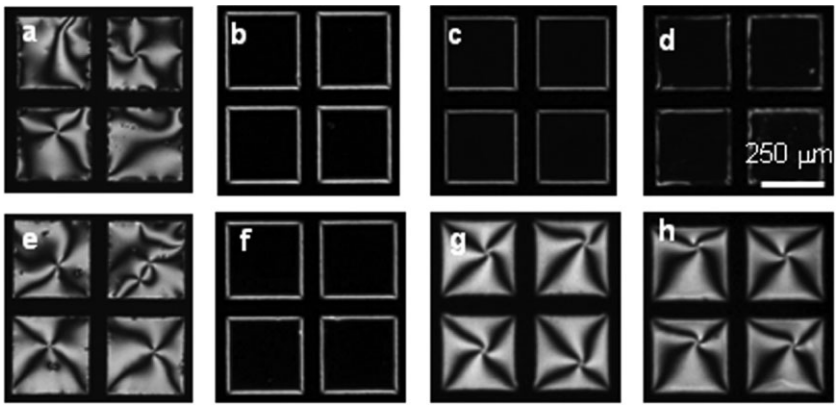


Figure 3. Optical response of 5CB to H^+ released from an enzymatic reaction. Copper grids with immobilized penicillinase were immersed into PBS buffer (pH = 7.0) containing different substrates. a) 1 mM penicillin G, b) no substrate, d) 1 mM penicilloate, e) 1 mM ampicillin, f) 1 mM tetraglycine, and g) 1 mM HCl. In some experiments, copper grids without immobilized penicillinase were used. These grids were immersed in PBS buffer containing c) 1 mM penicillin G and h) 1 mM HCl.

orientation of LCs. In another control experiment, we immersed the whole system into a solution containing penicilloate (a hydrolytic product of the enzymatic reaction), which was obtained by hydrolyzing penicillin G with penicillinase. Figure 3d shows that the image of LCs remains dark, suggesting that the presence of penicilloate does not affect the orientation of LCs.

2.3. Enzyme Specificity

An important consideration for the development of a biosensor is specificity. Thus, we immersed the LC pH sensor in buffer solutions containing ampicillin and tetraglycine (control), respectively. The former is also a substrate for penicillinase because it contains a β-lactam ring, which can be hydrolyzed by penicillinase. Figure 3e shows that the image of LC in the solution containing 1 mM of ampicillin becomes bright immediately after the immersion, while the one in the tetraglycine solution remains dark (Fig. 3f). Moreover, because the detection mechanism of the sensor is based on changes in pH, we also studied the response of the sensor to strong acids. Figure 3g shows that the image of the LCs becomes bright after the immersion of a penicillinase-modified grid into a solution containing 1 mM of HCl. However, when we immersed an unmodified copper grid (with impregnated LCs) into the same solution, the LCs also appeared bright (Fig. 3h), which is different from the dark appearance of the LCs when an unmodified grid was immersed in penicillin G solution (Fig. 3c). Thus, by comparing the results from the penicillinase-modified and unmodified grids, one can tell whether the solution contains penicillin G or strong acids.

2.4. Kinetics of Enzymatic Reactions

The above results demonstrate that our pH sensor can detect H^+ released from the hydrolysis of penicillin G before they are neutralized by the buffer solution. To determine the detection limit of the system, we compared the dynamic response of the LCs at different penicillin G concentrations. Figure 4a shows that the bright LC region slowly expands from the periphery to the center after the addition of 10 μM penicillin G. After 7 min, the bright region fills the entire square area. Furthermore, Figure 4a shows that when the concentration of penicillin G is lowered to 100 and 1 nM, respectively, the time required for the entire square area to appear bright also increases to 10 and 30 min, respectively. We can calculate the ratio of the bright area to the total square area as a function of exposure time for three different penicillin G concentrations as shown in Figure 4b. One can use the kinetic behavior of the response to estimate the concentration of penicillin G in the system. However, it is difficult to model the system because of two reasons. First, the diffusion length in the square region is too short (the length of each square is only 283 μm), which prevents us from collecting data over a long period of time. Second, the diffusion of H^+ occurs in a 2D square

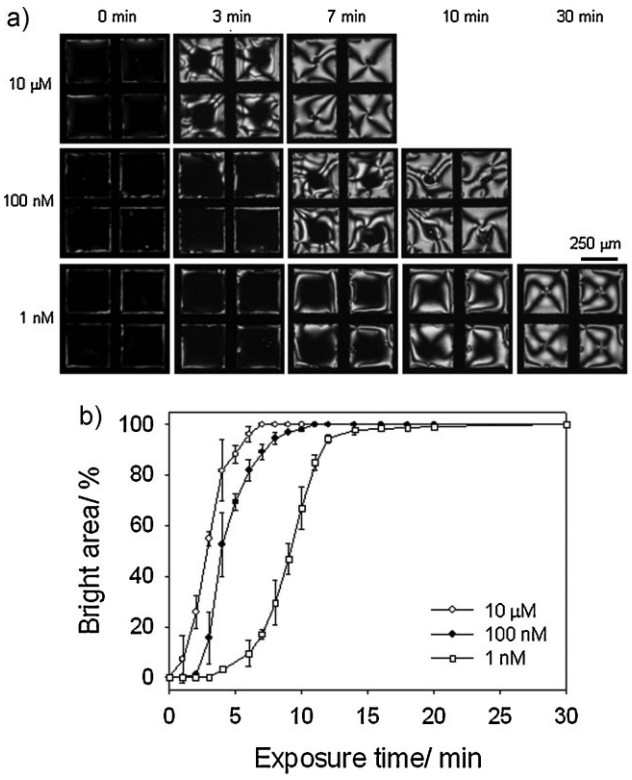


Figure 4. a) Evolution of optical images of 5CB (doped with 0.3% PBA and confined in penicillinase-modified copper grids) in PBS buffer containing different concentrations of penicillin G. Bright regions reflect the diffusion of H^+ released from the hydrolysis of penicillin G by the immobilized penicillinase. b) Estimated percentages of bright areas after different periods of exposure time.

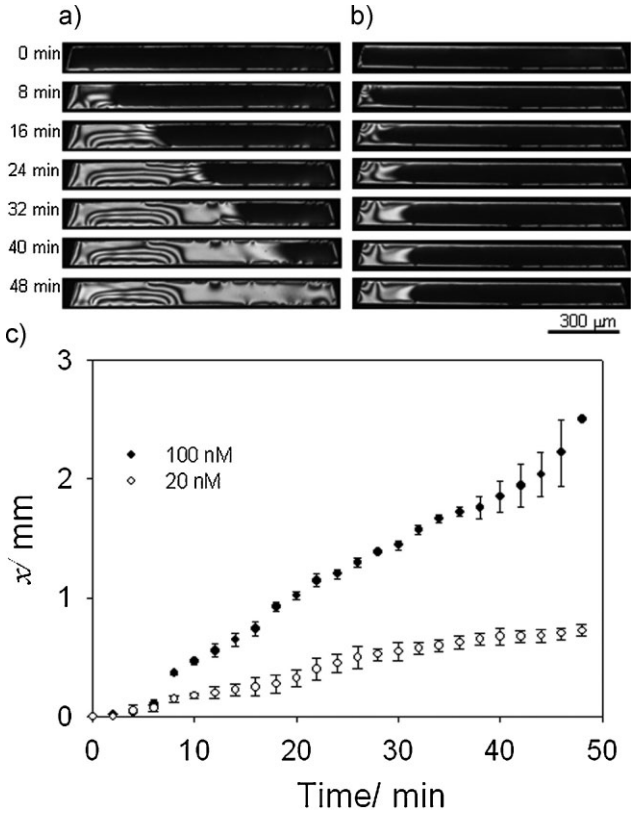


Figure 5. Optical response of 5CB (doped with 0.3% PBA and confined in bar-shaped, penicillinase-modified copper grids) after immersing the grids into PBS buffer containing a) 100 nM of penicillin G and b) 20 nM of penicillin G. c) Estimated length (x) of the bright bar (obtained from part a and b) as a function of exposure time.

geometry. To avoid these problems, we used a bar-shaped grid in the following experiments. We only modified one side of the metal bars of the grid with penicillinase. After we immersed the grid in 100 nM penicillin G solution, the optical image of the LCs became bright starting from the side with immobilized penicillinase, and then the bright region extended in the channel (Fig. 5a and b). This result suggests that the released H^+ diffuses in one direction within the channel. Figure 5c shows that the length of bright region increases with the exposure time at two different penicillin G concentrations. The results in Figure 5 allow us to model the kinetics of the enyzmatic reaction and the diffusion of H^+. However, we found that the diffusion distance of H^+ is larger than the experimental result (data not shown). The discrepency is probably caused by inhomogenity of the immobilized penicillinase layer or by the spatial constraints imposed on the penicillinase.[15,16] More efforts are needed to obtain a better model for this system.

3. Conclusions

In conclusion, we developed a highly sensitive and label-free pH sensor by using 5CB doped with PBA. As pH of the aqueous solution changes, orientations of the LCs undergo a homeotropic-to-planar or planar-to-homeotropic transition, which can be easily visualized as a dark or a bright image. The pH-driven optical response is attributed to the protonation and deprotonation of PBA at the aqueous/LC interface, which induces the orientational transitions of 5CB. The LC-based pH sensor shows great promise in monitoring enzymatic reactions. In our model system, the hydrolysis of β-lactam antibiotics catalyzed by penicillinase leads to the release H^+ which decreases pH in the vicinity of penicillinase-modified region and changes the optical appearance of the LCs. This sensor system shows a low detection limit (1 nM), real-time response, and good specificity. Because the detection principle based on localized pH change is general enough, this biosensor may also be suitable for detecting other types of enzymatic reactions which lead to changes in pH.

4. Experimental

Reagents: Glass slides were obtained from Marienfeld (Germany). DMOAP, poly(ethylene imine) (PEI, $M_w = 75\,000$), 50% glutaraldehyde aqueous solution, sodium cyanoborohydride ($NaBH_3CN$), sodium chloride (NaCl), penicillin G, ampicillin, penicillinase from *Bacillus Cereus* ($K_m = 60$ μM, $M_w = 28\,000$), PBA, biphenyl-4-carboxylic acid, lauric acid, and acetic acid were purchased from Sigma–Aldrich (Singapore). Sodium phosphate buffer (pH = 7.0), SDS (sodium dodecyl sulfate) was purchased from 1st base (Singapore). Liquid crystal 5CB was purchased from Merck (Singapore). All solvents used in this study were HPLC grades. Water was purified by using a Milli-Q system (Millipore, U.S.A.).

Immobilization of Penicillinase on Copper Grids: TEM copper grids (75 mesh, Electron Microscopy Sciences, U. S. A.) were first cleaned in methanol, ethanol, and acetone (sonication for 15 min in each solvent), and heated overnight at 100 °C to evaporate residual solvents. Next, the clean grids were immersed in an aqueous solution containing PEI (5 wt%). After 30 min, the grids were washed thoroughly with deionized water and dried in a 100 °C oven. Next, we immersed the grids in an aqueous solution containing glutaraldehyde (5 wt%) and sodium cyanoborohydride (10 mM) for 2 h. Finally, the grids were incubated in a buffer (which contained 0.5 mM sodium phosphate and 50 mM sodium chloride and was degassed for at least 1 h before use) containing penicillinase (0.2 mg mL^{-1}) for 12 h at 4 °C. For the bar-shaped grid, buffer solution (0.5 μL) containing penicillinase was dispensed onto one side of the grid. To prevent the evaporation of the penicillinase solutions, the grid was stored in a sealed and humid chamber for 12 h at 4 °C. Excess penicillinase was removed by incubating the grid in 2 × SSPE buffer (300 mM NaCl, 23 mM NaH_2PO_4, 2.8 mM ethylenediaminetetraacetic acid (EDTA) and 1% Triton X-100 for 10 min. All penicillinase-modified grids were kept at 4 °C before use.

Preparation of LC Samples: First, DMOAP-coated glass slides were prepared following a published procedure [17]. Briefly, glass slides were immersed in DMOAP solution (0.1 wt%) to obtain a layer of DMOAP on the surface. The glass slides were then cut into small squares (5 mm × 5 mm) and used as substrates for supporting the LCs. For fabrication of the LC-based pH sensor, unmodified copper grid was placed on the DMOAP-coated glass slide. Then, approximately 0.3 wt% PBA-doped 5CB (0.3 μL) was dispensed onto the grid, and excess LC was removed by using a capillary tube. Finally, the grid containing the LCs was covered with buffer solutions (300 μL) at different pH values. For monitoring enzymatic activities, the unmodified copper grid was replaced by a penicillinase-modified copper grid. Then, after filling the grid with PBA-doped 5CB, the grid was immersed in sodium phosphate buffer (300 μL, pH = 7.0) with different concentrations of penicillin G. The optical appearances and fluorescence images of these samples were observed by using polarizing optical and fluorescence microscopes, respectively [17].

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