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# Sensitive Detection of Trypsin using Liquid-Crystal Droplet Patterns Modulated by Interactions between Poly-L-Lysine and a Phospholipid Monolayer

Minmin Zhang and Chang-Hyun Jang*[a]

Liquid-crystal (LC) droplet patterns are formed on a glass slide by evaporating a solution of nematic LC dissolved in heptane. In the presence of an anionic phospholipid, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DOPG), the LCs display a dark cross pattern, indicating a homeotropic orientation. When LC patterns are incubated with an aqueous mixture of DOPG and poly-L-lysine (PLL), there is a transition in the LC pattern from a dark cross to a bright fan shape due to the electrostatic interaction between DOPG and PLL. Known to catalyze the hydrolysis of PLL into oligopeptide fragments, trypsin is preincubated with PLL, significantly decreasing the interactions between PLL and DOPG. LCs adopt a perpendicular orientation at the water–LC droplet interface, which gives rise to a dark cross pattern. This optical response of LC droplets is the basis for a quick and sensitive biosensor for trypsin.

## 1. Introduction

Proteases are enzymes that regulate and perform many essential biochemical processes in living organisms.[1] Highly selective and sensitive methods for the detection of specific proteases are needed for clinical diagnosis, environmental control, and the food industry.[2] Trypsin, which is derived from an inactive trypsinogen produced by pancreatic acinar cells, is one of the most important proteases in the digestive system.[3] Many different methods such as those involving specific chromogenic *p*-nitroanilide substrates,[4] optical nanoprobes,[5] surface-enhanced Raman scattering,[6] and fluorescence assays[7–10] have been used for monitoring the activity of trypsin. However, most of these methods require labeling of substrates and complex instrumentation to measure enzyme activity. Owing to these reasons, a simple, robust, and convenient approach to detect this protease is sought after.

Liquid crystal (LC)-based detection and sensing technologies have attracted considerable interest for the monitoring of various biochemical events due to their inherent specificity, simplicity, comparative low cost, rapid response, and amenability to miniaturization.[11–18] LC-based sensing is a typical example of a label-free technique[19] and requires neither substrate labeling nor complex instrumentation. In addition, it has high sensitivity because the orientation of LCs responds quickly to changes made at the LC interface, which in turn causes changes in their birefringence that can be observed under crossed polarizers.[20–24]

To date, several strategies for monitoring trypsin activity using LC-based sensing techniques have been published. In most of these systems, LCs have been confined within a copper grid supported on a glass slide.[20] As LCs are immiscible with water, the interactions between target molecules, such as phospholipids and other amphiphiles, and biomacromolecules, in aqueous solutions can be investigated at the LC–water interface. For example, Park et al. used LCs in a copper grid to detect trypsin activity in aqueous solutions.[21] They reported that a 17-amino-acid oligopeptide-based polymeric membrane at the LC–water interface retarded the transport of phospholipids to the interface, and that by incubating the membrane in an aqueous solution of trypsin that cleaved the oligopeptide-terminating lipids, the transport properties of the membrane could be enhanced. The distinct optical response of the LC verified the increased permeability of the oligopeptide-based membrane. However, this system had the disadvantages of lengthy sample preparation with consecutive washing steps.

Recently, we reported spontaneous formation of micrometer-scale LC droplet patterns in LCs supported on a solid surface.[25] The LC patterns exhibited a dark cross as an optical response when in contact with long-chain surfactant molecules, whereas they had a bright fan-shaped appearance in deionized water. The preparation of the surface-anchored LC patterns is simple and robust and is promising for the selective and sensitive investigation of a wide range of biochemical processes.

In this study, we investigated the feasibility of monitoring trypsin activity using LC droplet patterns. We observed that LCs in contact with 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DOPG) solutions formed a dark cross pattern representing a homeotropic orientation of the LC at the water–LC droplet interface. The pattern changed from a dark cross to a bright fan shape when an aqueous solution of poly-L-lysine (PLL) was transferred onto a self-assembled monolayer of DOPG at the water–LC droplet interface (Figure 1 A). This suggested that the electrostatic interaction between the negative-

[a] *M. Zhang, Prof. C.-H. Jang*
*Department of Chemistry, Gachon University*
*Seongnam-Si, Gyeonggi-Do 461-701 (Korea)*
*E-mail: chjang4u@gachon.ac.kr*

  

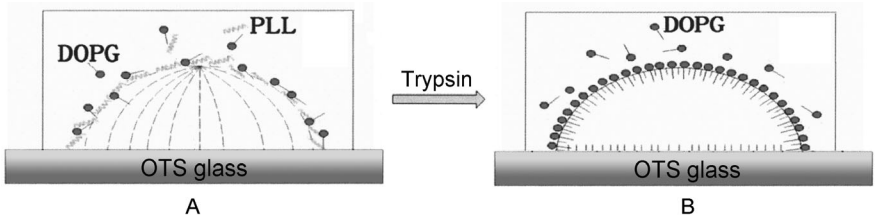


**Figure 1.** Orientational transitions of 5CB at the water–LC droplet interface. A) LC droplet pattern on immersion in an aqueous mixture of DOPG and PLL; B) the same procedure except PLL was preincubated with trypsin solution.

ly charged DOPG and the positively charged PLL had disrupted the alignment of the phospholipid membrane, which in turn caused changes in the LC pattern. This interaction was weakened in the presence of an enzyme that specifically cleaved PLL. An aqueous mixture of DOPG and PLL preincubated with trypsin transformed the optical response from a bright fan back to a dark cross, indicating a homeotropic orientation of LCs (Figure 1B). Using a simple thermodynamic model, these results indicate that trypsin can be detected using a biosensor based on LC droplet patterns. This LC-based sensing technique does not require protein labeling, tedious procedures, or complex instrumentation and is, therefore, simple, convenient, and robust.

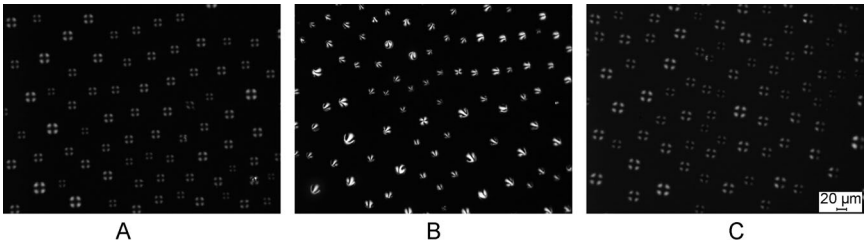


**Figure 2.** Polarized light microscopy images of surface-anchored LC droplet patterns A) when exposed to the air, and in contact with B) PBS solution, and C) an aqueous solution of DOPG (0.01 mM). Scale bar: 20 μm.

## 2. Results and Discussion

### 2.1. Optical Response of LC Droplet Patterns to Phospholipids

We recently reported the spontaneous formation of LC droplet patterns with large surface areas on glass microscope slides.[25] In this study, we investigated whether these LC droplet patterns could be used to monitor enzyme activity. Stable LC droplet patterns were prepared by spreading 4-cyano-4′-pentylbiphenyl (5CB, 1% v/v) dissolved in anhydrous heptane onto an octyltrichlorosilane (OTS)-treated glass slide. After the solvent evaporated, LC droplet patterns of micrometer-scale dimensions were formed on the slide surface. These patterns could be observed using polarized microscopy with a 10× objective lens. The small sizes of the patterns increase the sensitivity of the system because of their high surface-area-to-volume ratio. A dark cross appearance was obtained when the LC droplet patterns were exposed to air due to the homeotropic alignment of LCs at the air–LC droplet interface and LC–OTS interface (Figure 2A). In contrast, a bright fan-shaped image was observed when they were in contact with phosphate-buffered saline (PBS); this can be attributed to the planar orientation of LC molecules at the water–LC droplet interface (Figure 2B). Both these results are consistent with those previously reported, in which LC molecules had a perpendicular orientation at the air–LC interface and a planar orientation at the water–LC interface.[16] We also observed a dark cross pattern as a response to the LC droplet patterns being immersed into a DOPG solution (0.01 mM)—the homeotropic state of the LC patterns (Figure 2C). The dark appearance of LCs can be attributed to the thin monolayer of phospholipid, the long hydrocarbon chain of which penetrates the LC at the water–LC interface, as reported by Brake et al.[20] This is consistent with our previous observations that the organization of negatively charged amphiphilic phospholipids at the water–LC interface can be disrupted by electrostatic interactions with the positively charged PLL.[26] As PLL can be catalytically cleaved into smaller fragments by trypsin, incubating aqueous solutions of PLL with trypsin could eliminate or weaken the interactions of PLL with the DOPG membrane formed at the water–LC interface. Therefore, based on the two distinct patterns formed by LCs in aqueous solutions in the presence and absence of long-chain amphiphilic molecules, we predicted that it might be feasible to determine the enzymatic action of trypsin on PLL by observing the LC droplet pattern spread on an OTS-treated glass slide.

### 2.2. Monitoring the Enzymatic Reaction between Trypsin and PLL

First, we studied the interaction between the negatively charged DOPG and positively charged PLL. Figure 3A shows that LC droplet patterns immersed in the aqueous mixture of DOPG (0.01 mM) and PLL (0.1% w/v) exhibit a bright image, which is in contrast to the LCs without PLL (Figure 2C). This difference in the optical response indicates that there was a perturbation in the formation of self-assembled phospholipid monolayers at the water–LC interface, possibly due to the positively charged PLL. While the negatively charged DOPG causes 5CB to be aligned perpendicularly in the presence of PLL, the negative charges on DOPG interact with the positive charges on PLL and have a minimal effect on 5CB alignment. The pattern produced in the presence of both DOPG and PLL was

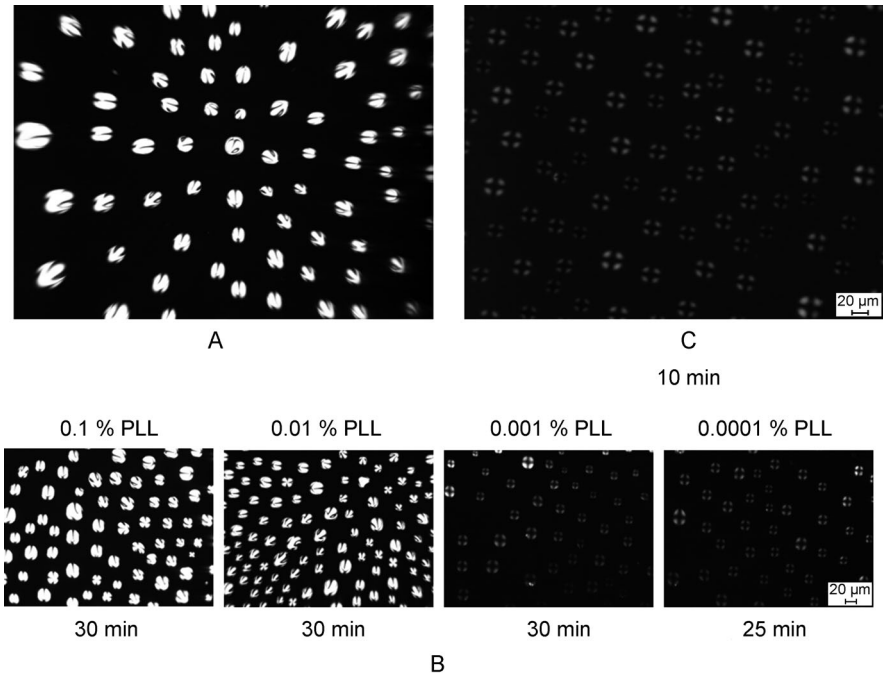


**Figure 3.** Polarized light microscopy images of LC droplet patterns in contact with an aqueous mixture of A) DOPG (0.01 mM) and PLL (0.1%), B) DOPG (0.01 mM) and PLL at varying concentration, and C) DOPG (0.01 mM) and PLL (0.001%) preincubated with trypsin for 2 h. Scale bars: 20 μm.

bright fan-shaped, similar to the LC pattern formed in contact with PBS solution, and not a dark cross, suggesting that PLL counteracts the effect of DOPG on droplet patterns.

In our previous study, we reported that the strength of the interaction between PLL and DOPG was dependent on the concentration of PLL and the incubation time.[27] In order to find the optimum concentration of PLL for this detection system, a series of mixtures of DOPG (0.01 mM) and varying concentrations of PLL were transferred onto the LC droplets and observed for 30 min. When 1 μL of a solution of DOPG (0.01 mM) and PLL at concentrations of 0.1% and 0.01% was introduced onto the LC droplets, the bright fan-shape pattern was obtained, indicating a planar orientation of the LCs at the water–LC droplet interface. However, when the PLL concentration was $\leq$0.001%, the LC droplet began to darken after 30 min or less, corresponding to a homeotropic alignment of LCs at the water–LC droplet interface (Figure 3B). These results suggest that decreasing the PLL concentration shortens the time for the optical response to occur. The concentration of PLL also affected the orientation of LC molecules at the water–LC interface. These findings indicate a critical concentration of 0.001% PLL for this detection system. At concentrations below 0.001%, the positively charged PLL initially neutralizes the negatively charged DOPG and a bright fan-shaped pattern is observed. However, the interaction between PLL and DOPG is too weak to prevent the LC alignment of DOPG. Thus, the bright fan-shaped pattern persists until the number of DOPG molecules present at the water–LC droplet interface is sufficient to cause perpendicular orientation of the LCs, resulting in an optical change.

Trypsin selectively cleaves peptide bonds at the C-terminal side of lysine and arginine. A previous study had demonstrated that trypsin enzymatically hydrolyzes PLL, therefore we hypothesized that the interaction between DOPG and PLL could be weakened or eliminated by preincubating the PLL solution with an aqueous solution of trypsin.[27] We chose a PLL concentration of 0.001% and an observation time of 30 min for the detection of trypsin activity because higher concentrations of PLL might have decreased the sensitivity of the detection system. We incubated a PLL solution (0.001%) containing trypsin (0.1 mg mL$^{-1}$, pH 7.4) for 2 h at room temperature before mixing it with DOPG (0.01 mM). Upon immersion into this mixture, the LC droplets changed from bright fan patterns to dark crosses within 10 min (Figure 3C), indicating a homeotropic state of the LCs at the water–LC droplet interface. Enzymatic hydrolysis of PLL by trypsin reduces the electrostatic interaction between DOPG and PLL and enables DOPG molecules to self-assemble and penetrate into the LCs at the water–LC droplet interface, resulting in a dark cross image that is correlated to the homeotropic state of LCs. These results suggest that the enzymatic action of trypsin on PLL can be monitored using LC droplet patterns.

### 2.3. Specificity and Sensitivity of the Detection Platform with the LC Droplet Patterns

To further characterize the orientational transition of 5CB patterns resulting from the enzymatic cleavage of PLL by trypsin in the aqueous phase, a series of control experiments comparing PLL and other proteins were performed using the trypsin–chymotrypsin inhibitor from *Glycine max* (Bowman–Birk inhibitor from soybean). The Bowman–Birk inhibitor is a monomeric protein possessing two independent subdomains for the binding of trypsin and α-chymotrypsin. The inhibitor forms a 1:1 complex with trypsin or chymotrypsin,[28] blocking their activity. First, we added the trypsin–chymotrypsin inhibitor to the solution of trypsin (1 mg mL$^{-1}$) such that the enzyme/inhibitor molar ratio was 1:6. This solution was incubated for 10 min at room temperature, then for a further 2 h at room temperature with the PLL solution prior to mixing with DOPG. The LC droplets on the glass slide were then immersed into the aqueous solution of DOPG and PLL preincubated with trypsin and the inhibitor. In contrast to the DOPG–PLL mixture with trypsin

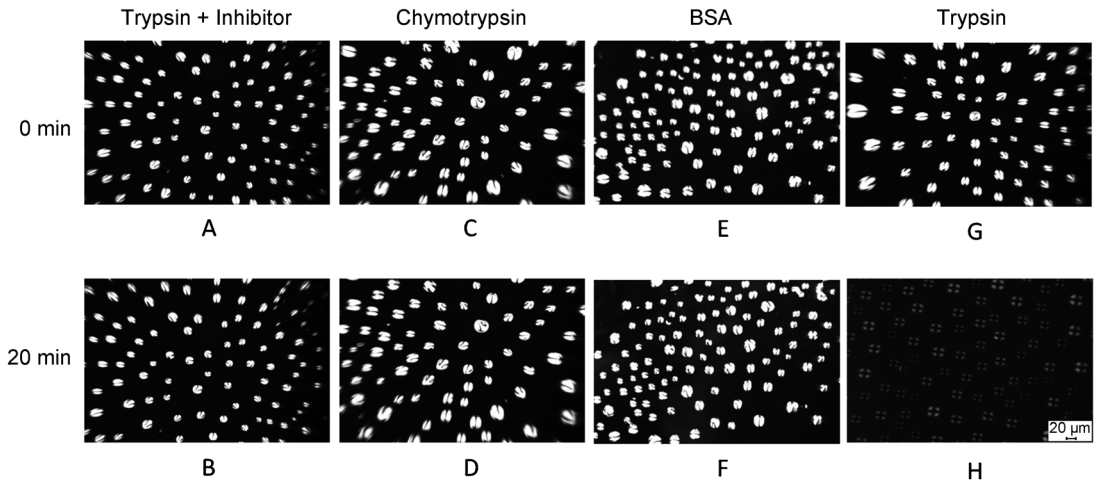


**Figure 4.** Polarized light microscopy images of LC droplet patterns in contact with an aqueous mixture of DOPG (0.01 mM) and PLL (0.001 %) preincubated with 0.1 mg mL$^{-1}$ of A, B) trypsin and inhibitor, C, D) chymotrypsin, E, F) BSA, or G, H) trypsin for 2 h at room temperature. Micrographs A, C, E and G indicate the patterns at 0 min and B, D, F and H indicate the patterns after 20 min. Scale bars: 20 μm.

alone, the mixture of DOPG, PLL, trypsin, and the inhibitor did not cause an orientational transition of the 5CB pattern to a homeotropic alignment after 20 min of contact, and the LC retained the bright fan shape (Figure 4 A and B). We also performed two control experiments with chymotrypsin and bovine serum albumin (BSA). Neither chymotrypsin (which preferentially cleaves on the carboxyl side of an aromatic amino acid) nor BSA can catalyze the cleavage of PLL, and did not elicit a change in the 5CB pattern. Accordingly, the LCs maintained a planar orientation at the water–LC droplet interface, exhibiting a bright appearance under optical microscopy (Figure 4 C–F). These results, on the whole, suggest that the transition in LC patterns observed in the presence of trypsin arises from the selective cleavage of PLL by trypsin rather than binding or nonspecific interactions.

After confirming the specificity of our method for enzyme detection, we sought to determine the detection limit for trypsin activity. To accomplish this, a solution of PLL (0.001 %) was mixed with trypsin at different concentrations (10, 1, 0.1 and 0.01 μg mL$^{-1}$) and preincubated for 2 h at room temperature prior to mixing with DOPG. These solutions were then transferred onto the LC droplets, and the optical response of the LCs was observed after 10 min. The LC droplet pattern showed a dark cross appearance when the trypsin concentration was $\geq$0.1 μg mL$^{-1}$, indicating that the LCs adopted a homeotropic state at the water–LC droplet interface above this concentration (Figure 5 A–C). However, we found no change in the pattern at the lower trypsin concentration of 0.01 μg mL$^{-1}$ (Figure 5 D), suggesting a planar anchoring transition of the LCs at the water–LC droplet interface. This observation suggests that, at low concentrations of trypsin, there was enough uncleaved PLL remaining to react with DOPG. Hence, the results indicate that the detection limit of trypsin in this system is around 0.1 μg mL$^{-1}$. Compared to our previous studies on trypsin, the detection limit in this droplet pattern system is 10 times lower than that of LC in the liquid phase,[26] indicating a tenfold increase in the sensitivity of the new system. The increased surface density of functional molecules at the LC droplet pattern interface and gradual evaporation of water from the aqueous mixture might contribute to this increase in sensitivity.

## 3. Conclusion

LC droplet patterns were prepared by spreading a solution of 5CB in heptane onto OTS-treated glass slides and allowing the heptane to evaporate at room temperature. Electrostatic interactions between the negatively charged DOPG and positively charged PLL could be observed using optical microscopy as

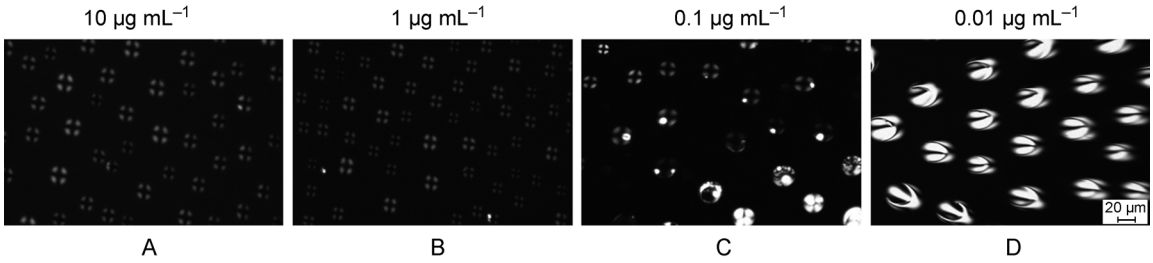


**Figure 5.** Polarized light microscopy images of LC droplet patterns contacted with the aqueous mixture of DOPG (0.01 mM) and PLL (0.001 %) preincubated with varying concentrations of trypsin for 2 h: A) 10 μg mL$^{-1}$, B) 1 μg mL$^{-1}$, C) 0.1 μg mL$^{-1}$, and D) 0.01 μg mL$^{-1}$. Scale bar: 20 μm.

a result of the orientational transitions of LC droplet patterns. The optical response of LC droplet patterns to addition of an aqueous mixture of DOPG and PLL at different concentrations was also investigated. The electrostatic interaction was weakened by incubating PLL solutions with trypsin, which can hydrolyze PLL. Experiments were also performed to determine the detection limit of trypsin, which was found to be 0.1 $\mu g\,mL^{-1}$ for this system. Compared with previously reported LC-based sensing approaches, this simple method has improved the detection sensitivity by tenfold. These results suggest that LC droplet patterns can be used for the development of simple and sensitive detection of enzymatic reactions. Thus, we have successfully developed a sensitive method to detect the enzymatic activity of trypsin by observing micrometer-scale LC droplet patterns.

## Experimental Section

### Materials

Premium glass microscope slides were obtained from Fisher Scientific (Pittsburgh, PA). Nematic LC 5CB was purchased from EM Industries (Hawthorne, NY). Sulfuric acid ($\geq$95% w/w), $H_2O_2$ (30% w/v) , OTS, DOPG sodium salt, PLL (0.1% w/v, 150 000–300 000 $mol\,wt^{-1}$), trypsin, trypsin–chymotrypsin inhibitor from *G. max* (soybean), and PBS [sodium phosphate (10 mM), NaCl (138 mM), KCl (2.7 mM), pH 7.4] were purchased from Sigma–Aldrich. *n*-Heptane (anhydrous) was purchased from Daejung Chemicals and Metals Co. Ltd. (Siheung, South Korea). All aqueous solutions were prepared with deionized water (18 $M\Omega\,cm^{-1}$) using a Milli-Q water purification system (Millipore, Bedford, MA).

### Treatment of Glass Microscope Slides with OTS

Glass microscope slides were cleaned according to a previously published procedure.[29] In brief, the slides were immersed in piranha solution (sulfuric acid/$H_2O_2$, 7:3 v/v; warning: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store the solution in closed containers) for 30 min at approximately 80 °C. The slides were then rinsed sequentially with water, ethanol and methanol, and dried under a stream of nitrogen gas, after which they were heated at 120 °C overnight prior to OTS deposition. The piranha-solution-cleaned slides were then immersed in an OTS solution (0.5 mM in heptane) for 30 min at room temperature. The samples were then rinsed with $CH_2Cl_2$ and dried under nitrogen. Next, 5CB was introduced between the slides and the resulting optical texture was examined using polarized light to confirm homeotropic anchoring. Any sample not exhibiting homeotropic anchoring of 5CB was rejected.

### Preparation of Aqueous Solutions of Phospholipids (DOPG)

DOPG was dissolved in $CHCl_3$ (50 $mg\,mL^{-1}$), after which the solvent was removed by evaporation under nitrogen and subsequent desiccation under vacuum (3 h). The dried lipid film was resuspended in PBS to generate a multilamellar vesicle suspension. The suspension was then sonicated to clarity using a probe sonicator and filtered through a 0.20 μm filter prior to use.

### Preparation of Optical Cells to Form LC droplet patterns

Micrometer-scale LC droplet patterns supported on OTS-treated glass slides were formed by spotting 5CB (1% in anhydrous heptane, v/v, 1 μL) onto the glass surface. Next, aqueous solutions of interest (1 μL) were transferred onto the LC droplets at room temperature. All aqueous solutions coupled to enzymatic reactions were prepared in PBS (10 mM, pH 7.4).

### Optical Examination of LC Textures

The optical images of LCs were obtained using the polarized light of an optical microscope (Eclipse LV100POL; Nikon, Tokyo, Japan). The images were captured using a digital camera (DS-2Mv; Nikon, Tokyo, Japan) and a microscope with a 10× objective lens. The camera had a resolution of 1600×1200 pixels, a gain of 1.00× and a shutter speed of 0.1 s.

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**Keywords:** biosensors · liquid crystals · orientational transition · poly-L-lysine · trypsin

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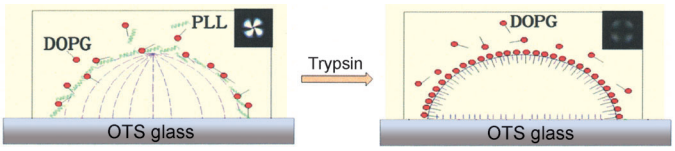


**Counting crosses:** Liquid crystals (LCs) of 4-cyano-4′-pentylbiphenyl form a bright fan shape (planar orientation at the water–LC interface) when in contact with a mixture of 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DOPG) and poly-L-lysine (PLL). The LCs take on a dark cross appearance (homeotropic orientation) in a DOPG–PLL mixture pre-incubated with the protease trypsin. This optical response can be used to measure trypsin activity. OTS = octyltrichlorosilane.

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