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Liquid crystal-based detection of thrombin coupled to interactions between a polyelectrolyte and a phospholipid monolayer

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ABSTRACT

Herein, we describe a real-time, label-free biosensing strategy for thrombin detection that uses the orientational properties of nematic liquid crystals (LCs) and the interactions between a polyelectrolyte and a phospholipid monolayer. The imaging principle is based on the disruption of the orientation of 4-cyano-4′-pentylbiphenyl by reorganized 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (DOPG) at the aqueous/LC interface. Positively charged, multiple arginine peptides (poly-L-arginine hydrochloride) interacted with negatively charged DOPG at the aqueous/LC interface, which caused reorganization of the phospholipid layer and induced an orientational transition of LCs from a homeotropic to a planar state. As a result, a dark to bright shift in the optical response was observed. Thrombin cleaves poly-L-arginine hydrochloride into peptides. Thus, when thrombin was added, the optical signals generated by the LCs reverted from bright to dark because of the weakened ability of the fragments to induce electrostatic interactions. The limit of detection of the LC-based sensor was 0.25 ng/mL (6.7 pM) thrombin, and the sensor was fully reusable. The detection limit of our LC-based interface sensor is 600 times lower than that of a previously reported enzyme-linked aptamer assay for the detection of thrombin. Thus, we have established a new, simple thrombin biosensor with high sensitivity and low interference.

Phospholipids, the most abundant lipids in cell membranes, are used in a wide range of applications for the detection and characterization of biomolecular interactions, including biological sensing, transmembrane transport, receptor interactions, and cellular signaling [1–4]. Because of their amphiphilic nature, phospholipids form spherical lipid bilayers known as liposomes and vesicles. When phospholipid vesicles are exposed to a hydrophobic surface, they fuse with the surface and form a planar membrane, resulting in the formation of a lipid monolayer [2,5,6].

In recent years, liquid crystalline materials used as material interfaces to biomolecular events have been explored. Biomolecular events at the interface of a liquid crystals (LC)[1] can trigger ordering transitions in LCs that are readily visible to the naked eye by using polarized microscopy [7–9]. Previous studies have demonstrated that the presence and organization of amphiphiles at the interface between the LCs and an immiscible aqueous phase can affect the orientational ordering of the LCs. Brake et al. reported that contact with the interface of a thermotropic LC with an aqueous solution of phospholipid resulted in the spontaneous assembly of a monolayer of phospholipid at the aqueous/LC interface, corresponding to a dark image in the optical response. On protein binding and enzyme activity at these phospholipid-laden interfaces, Brake and co-workers observed that the LC undergoes an ordering transition from the homeotropic state to the planar state, which causes a dark to bright optical change [9].

Previously, Park and Abbott reported on the use of phospholipid membranes decorated with peptide amphiphiles to monitor interfacial enzymatic reactions at an aqueous/LC interface [10]. An amino acid oligopeptide immobilized at the aqueous/LC interface led to an ordering transition of the LCs. A combination of chemical and physical interactions between the amino acid oligopeptide and the lipid-laden interface caused reorganization of the monolayer. Subsequently, exposure to an enzyme that cleaved the immobilized oligopeptide substrate induced the LCs to revert from a tilted orientation to a homeotropic orientation. The results of this study suggested that oligopeptide-decorated liquid crystals could be used to selectively monitor enzymatic activity.

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[1] *Abbreviations used:* LC, liquid crystal; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt; ELONA, enzyme-linked oligonucleotide assays; ELISA, enzyme-linked immunosorbent assays; PLA, poly-L-arginine hydrochloride; 5CB, 4-cyano-4′-pentyl-biphenyl; PBS, phosphate-buffered saline; OTS, octyltrichlorosilane; DI water, deionized water; ELAA, enzyme-linked aptamer assay.

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Thrombin, a serine protease, plays a significant role in the coagulation cascade, thrombosis, and hemostasis [11–13]. In addition, thrombin also plays a major role in vasospasms, which often result in cerebral ischemia and infarction [14,15]. Detecting a specific protease such as thrombin sensitively and selectively is very important for clinical diagnosis and therapeutic research [16,17]. For example, thrombin can be used as a biomarker for tumor diagnosis. Studies have described the detection of thrombin with "hot spots," enzyme-linked oligonucleotide assays (ELONA), and enzyme-linked immunosorbent assays (ELISA) [18–20]. However, to the best of our knowledge, no one has used changes in the orientation of liquid crystals coupled to interactions between a polyelectrolyte and a phospholipid layer to detect thrombin enzymatic activity.

In this study, we developed a simple and convenient strategy to image biological interactions using polyelectrolyte-disrupted phospholipid membranes supported on an aqueous/LC interface. We predicted that the interaction between positively charged poly-L-arginine hydrochloride (PLA) and negatively charged 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (DOPG) accounts for reorganization of the phospholipid membrane and leads to a homeotropic to planar ordering transition of the LCs at the interface (Fig. 1A). Conversely, these interactions would be changed by incubation of PLA with a protease specific for the polyelectrolyte substrates. As a result, the arrangement of the phospholipid membrane was restored, leading to ordering transitions of the LCs from a planar to a homeotropic state (Fig. 1B), thereby allowing real-time, label-free investigation of the surface. Because thrombin selectively hydrolyzes PLA peptides into fragments, the enzymatic reaction between thrombin and PLA was used to confirm our prediction.

Experimental

Materials

Premium glass microscope slides and 8-well chamber slides were obtained from Fisher Scientific (Pittsburgh, PA). Nematic liquid crystal 4-cyano-4′-pentyl-biphenyl (5CB) manufactured by BDH was purchased from EM Industries (Hawthorne, NY). Copper specimen grids (50 meshes, pitch 500 μm, hole 420 μm, bar 80 μm, thickness 25 ± 5 μm) were obtained from Gilder Grids (Grantham, UK). Sulfuric acid, hydrogen peroxide (30% w/v), octyltrichlorosilane (OTS), thrombin, DOPG, poly-L-arginine hydrochloride (PLA) (mol wt 15,000–70,000), and phosphate-buffered saline (PBS) (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl; pH 7.4) were all from Sigma–Aldrich. Otherwise noted, all aqueous solutions were prepared with deionized water (18 MΩ cm) using a Milli-Q water purification system (Millipore, Bedford, MA).

Treatment of glass microscope slides with OTS

Glass microscope slides were cleaned in accordance with the procedure detailed in a previous publication [21]. Briefly, the slides were immersed in piranha solution (70% (v/v) sulfuric acid and 30% (v/v) hydrogen peroxide) for 30 min at approximately 80 °C. (Warning: since piranha solution reacts strongly with organic compounds. It should be handled with extreme caution; DO NOT store the solution in closed containers.) The slides were then rinsed with water, ethanol, and methanol and dried under a stream of gaseous nitrogen. The sliders were then stored at 120 °C overnight before OTS deposition. OTS solution (0.5 mM) was prepared, and the piranha-cleaned slides were immersed in 0.5 mM OTS in heptane solution for 30 min at room temperature. Following OTS deposition, the slides were rinsed with methylene chloride 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.

Formation of a self-assembled monolayer of phospholipids (DOPG)

DOPG was solubilized in chloroform (50 mg/mL), and 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 before use. The phospholipid monolayer was formed by touching the copper grid impregnated with 5CB to the phospholipid solution in the optical cell.

Preparation of glass slide-supported optical cells

OTS-treated glass slides were fixed to the bottom of an 8-well chamber slide with epoxy, and transmission electron microscopy grids were placed onto the OTS-coated glass slides. Subsequently, 2.0 μL of 5CB, heated to 45 °C (isotropic phase of 5CB, >35 °C),

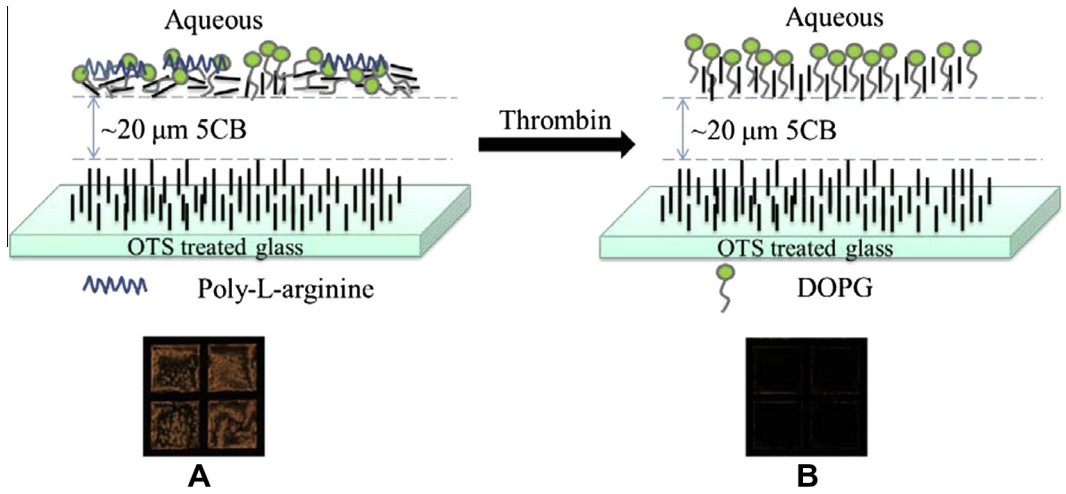

Fig.1. Schematic illustration of the orientational transitions of 5CB before and after the enzymatic reaction between thrombin and poly-L-arginine: (A) planar orientation and (B) homeotropic orientation.

was dispensed onto each grid, and the excess liquid crystal was removed using a 20-μL capillary tube. Subsequently, 400 μL of PLA solution was quickly introduced into the well with a pipette. Each assay was performed at least six times independently.

Optical examination of LC textures

Optical images of LCs were obtained under polarized light with an optical microscope (Eclipse LV100 POL; Nikon, Tokyo, Japan). The optical images were captured using a digital camera (DS-2Mv; Nikon, Tokyo, Japan) and a microscope with a 4× objective lens. The camera had a resolution of 1600 × 1200 pixels, a shutter speed of 1/10 s, and a gain of 1.00×.

Results and discussion

Imaging interactions between poly-L-arginine and phospholipids at the aqueous/LC interface

5CB appeared bright when it was in contact with DI water (Fig. 2A), indicating a tilted or planar orientation of the LCs at the aqueous/LC interface. To establish the polyelectrolyte-disrupted phospholipid membrane system with which to observe the enzymatic activity of thrombin, we first formed a self-assembled monolayer of DOPG at the aqueous/LC interface. The optical appearance of the LCs gradually changed from bright to dark (Fig. 2B) within 2 min, which corresponded to a planar to homeotropic (perpendicular) shift in the orientation of the LCs.

After contact with the DOPG solution with the interface of a LC in the optical cell for 30 min, a stable, spontaneous assembled monolayer of phospholipids was obtained. We then washed the DOPG solution with PBS five times to remove excess phospholipids from the bulk solution. Subsequently, the PBS solution was replaced with an aqueous solution of 0.064 mg/mL PLA in PBS. The arginine peptide was positively charged at pH < 11 because of the high isoelectric point of arginine. Bright domains, observed within 30 s (Fig. 2C), started to nucleate (Fig. 2D) and grow (Fig. 2E) at the LC interface. These results suggested that electrostatic interactions between positively charged PLA and negatively charged DOPG disrupted the integrity and organization of the lipid monolayer at the LC interface, thereby inducing an orientational transition of the LCs from a homeotropic to a planar state. According to our previous study, the time-dependent nucleation and growth of bright domains in the LCs might relate to the dynamic lateral distribution of PLA–DOPG complexes at the LC interface [22].

Monitoring the enzymatic reaction of thrombin with PLA

Thrombin is an enzyme that is known to enzymatically hydrolyze PLA peptides into fragments. It was hypothesized that the interaction between PLA and DOPG would, therefore, be weakened by incubation of PLA with thrombin. First, we tested the optical response of the LCs after transferring an aqueous solution of 0.032 mg/mL PLA onto the LC interface decorated with a DOPG monolayer. The optical appearance changed from dark (Fig. 3A) to bright (Fig. 3B) within 3 min, indicating that the LCs underwent an ordering transition from a homeotropic state to a planar state. We then exchanged the aqueous solution of PLA with PBS five times to remove free PLA from the interface. During the washing process, aggregation of bright domains was observed, as described above (not shown in the figure).

Next, the PBS solution in the optical cell was replaced by an aqueous solution of 2.5 ng/mL thrombin. Indeed, the size of the bright domains gradually decreased thereafter. Within 30 min, the LCs appeared uniformly black (Fig. 3C), which indicated that the orientation of the LCs had reverted to a complete homeotropic state. Because PLA bound to the phospholipid membrane was

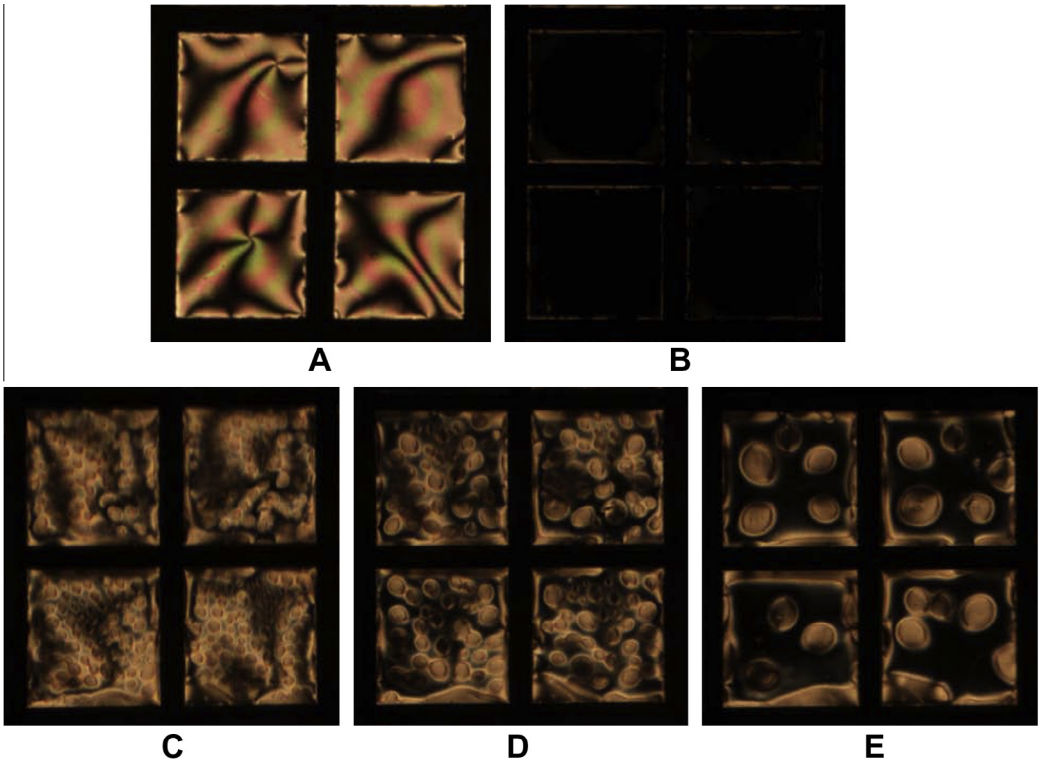

Fig.2. Polarized light microscopy images of LCs: (A) in contact with DI water; (B) after 2 min of contact with an aqueous dispersion of DOPG; and (C–E) in contact with the DOPG-decorated interface and 0.064 mg/mL PLA for (C) 30 s, (D) 90 s, and (E) 30 min.

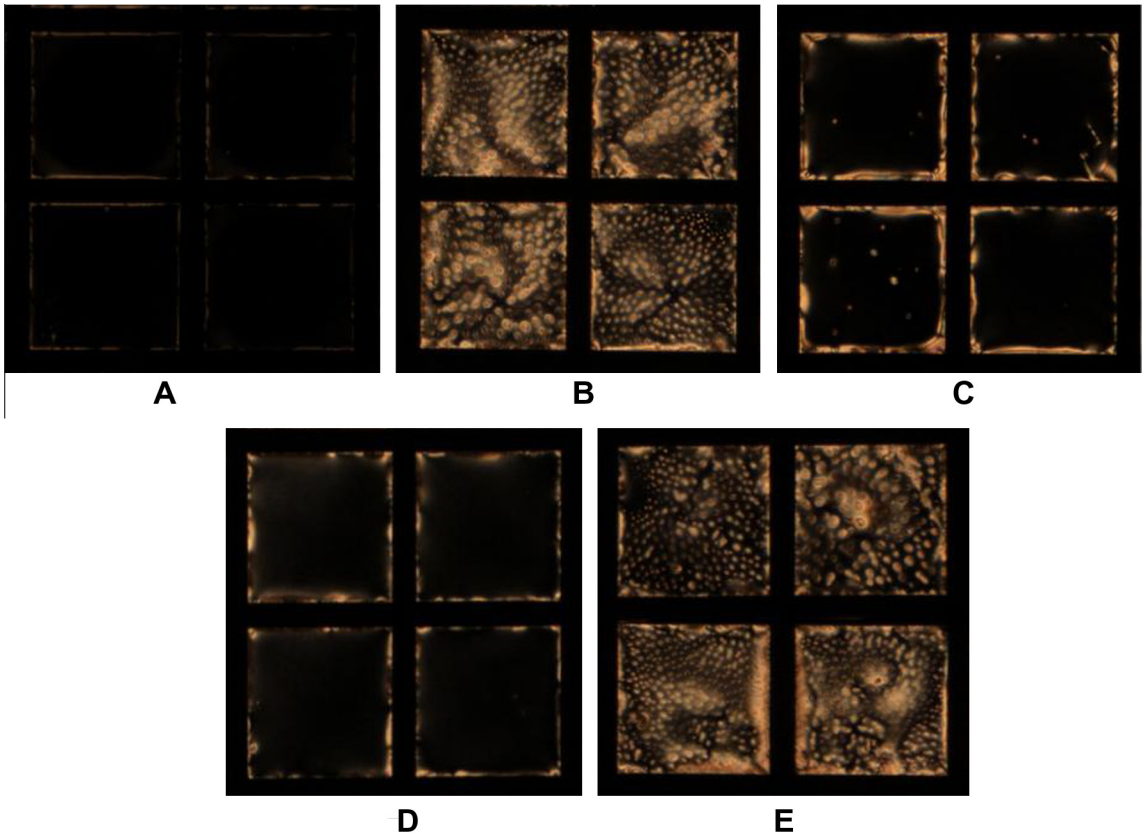

Fig.3. Polarized light microscopy images of LCs: (A) after 3 min of contact with an aqueous dispersion of DOPG; (B) 3 min after transferring 0.032 mg/mL PLA onto the DOPG-decorated interface, followed by (C) contact with an aqueous solution of thrombin for 30 min; and (D) 30 min after immersion of the DOPG-decorated interface into an aqueous solution of thrombin, followed by (E) contact with an aqueous solution of 0.032 mg/mL PLA for 3 min.

enzymatically cleaved by thrombin into small polyelectrolyte fragments that desorbed from the aqueous/LC interface into the bulk solution, the integrity and arrangement of the phospholipid monolayer, previously disrupted by PLA, were restored. Thus, the orientation of the LCs changed from a planar to a homeotropic state. Fig. 3 reveals that the LC is bright near the metal surface. The bright rims are due to the interaction of the LC with the grid surface. Previous study has noted that when LCs are confined to grids, the regions of LC near the grid surface modulated between maximum transmission and maximum extinction. The anchoring of 5CB at the grid surfaces is homeotropic and the extent of alignment of 5CB on the grid surface is small compared to the grid spacings [23]. Thus we conclude that the textures of LC confined within the grid are dominated by interactions at the LC interface not the grid surface.

To further address the feasibility of using this system to monitor the enzymatic reaction between thrombin and PLA, two control experiments were performed. First, a DOPG-decorated LC interface was immersed under an aqueous solution of 2.5 ng/mL thrombin in PBS; the optical response of the LCs remained dark (Fig. 3D), which indicated that the addition of the thrombin solution did not cause an orientational transition of the LCs at the LC interface. Second, the aqueous solution of thrombin was exchanged with PBS five times to wash the interface and was then replaced by an aqueous solution of 0.032 mg/mL PLA. The optical appearance of the LCs immediately changed from black to bright (Fig. 3E) within 3 min, which demonstrated that the orientation of the LCs changed from a homeotropic state to a planar state. These results suggested that the enzymatic reaction of thrombin with PLA peptides could be monitored through the interactions between positively charged PLA and negatively charged DOPG at the aqueous/LC interface.

Another two enzymes, lipase and lysozyme, were applied to thoroughly evaluate the specificity of the interaction. The procedure was the same as that shown for thrombin. In contrast to the results obtained for thrombin, the optical texture of 5CB remained bright after 30 min of contact with an aqueous solution of lipase and lysozyme (figures not shown), indicating that the anchoring of 5CB remained planar. The sample incubated with lipase and lysozyme did not show any orientational transition to homeotropic alignment after 30 min. These results suggest that the interaction between PLA and phospholipid membrane can be controlled by thrombin in a way that prevents PLA from interacting with DOPG by enzymatic activities at the interface of 5CB.

Optimization of PLA concentration

After confirming the feasibility of our method for the enzymatic detection of thrombin, we then explored the enzymatic reaction between thrombin and PLA peptides in more detail. Because higher concentrations of PLA might decrease the sensitivity of our experimental system, we assessed the optimal concentration of PLA in order to maximize performance.

First, we sought to find the detection limit of PLA to determine the optimal concentration with which to measure thrombin activity with high spatial resolution. We introduced an aqueous solution of 0.064 mg/mL PLA onto the DOPG-decorated LC interface; the optical appearance of 5CB became bright within 3 min (Fig. 4A). The LCs also displayed a uniformly bright appearance after the DOPG-decorated LC interface was immersed in an aqueous solution of 0.032 mg/mL PLA for 30 min (Fig. 4B). However, the optical texture of the LCs was partially bright after an aqueous solution of 0.0064 mg/mL PLA was transferred onto the

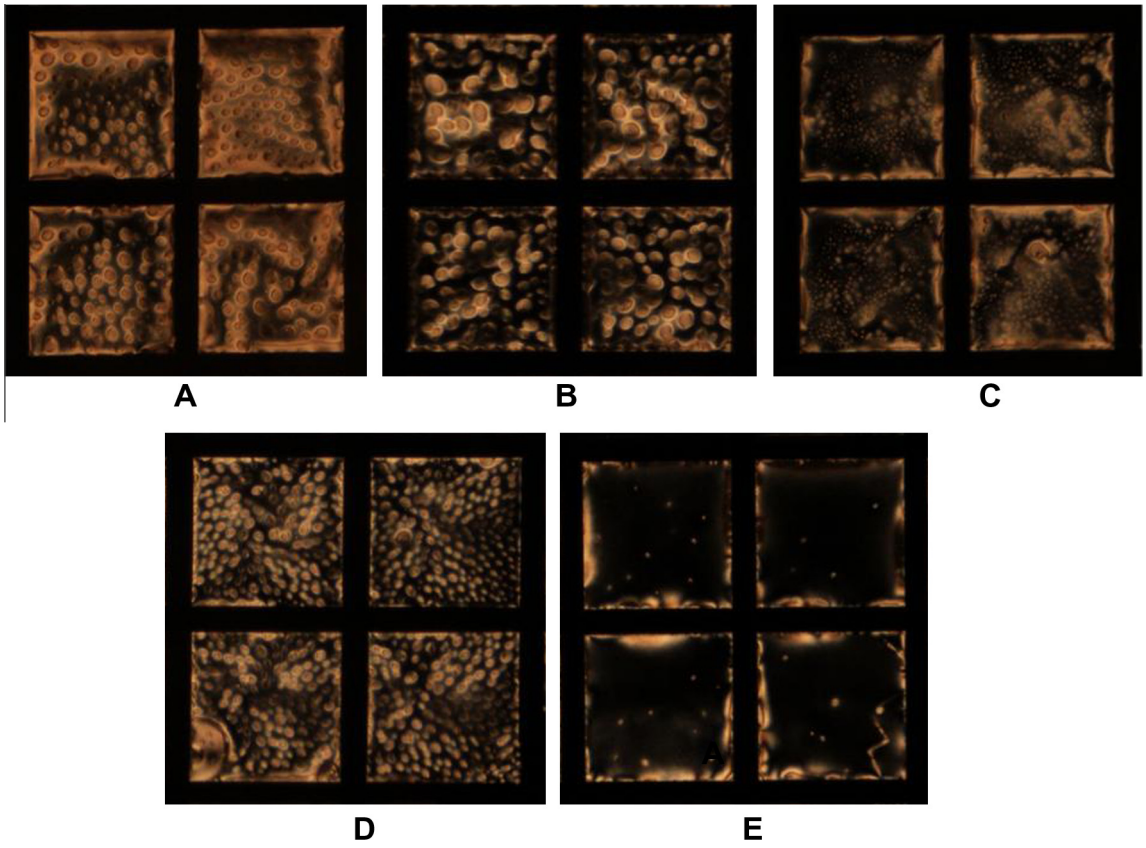

Fig.4. Polarized light microscopy images of LCs supporting a DOPG monolayer at the interface: (A) 3 min after contact with an aqueous solution of 0.064 mg/mL PLA; (B) 30 min after contact with an aqueous solution of 0.032 mg/mL PLA; (C) 1 h after contact with an aqueous solution of 0.0064 mg/mL PLA; (D) after 3 min of contact with an aqueous solution of 0.032 mg/mL PLA; and (E) in contact with an aqueous solution of thrombin for 30 min.

DOPG-decorated LC interface for 1 h (Fig. 4C). From these observations, we elected to use an aqueous solution of PLA with a concentration of 0.032 mg/mL to monitor the enzymatic activity of thrombin.

We also evaluated the reutilization of phospholipids at the interface. We hypothesized that the phospholipid membrane formed at the aqueous/LC interface could be reused after the enzymatic reaction between thrombin and PLA. As soon as the optical appearance of the LCs became uniformly black after the introduction of an aqueous solution of 2.5 ng/mL thrombin, we exchanged the aqueous solution with PBS five times to wash away the excess thrombin and free polyelectrolyte fragments in the optical cell. Next, we transferred an aqueous solution of 0.032 mg/mL PLA onto the DOPG-decorated LC interface again. The optical texture of 5CB changed from dark to bright within 3 min (Fig. 4D), indicating that the orientation of 5CB changed from a homeotropic to a planar state. Subsequently, after the readdition of thrombin, the LCs changed from bright to almost dark within 30 min (Fig. 4E), representing that the orientation of the LCs reverted from a planar to a homeotropic state. The phospholipid membrane generated at the aqueous/LC interface can perform at least 3 detection cycles and still retain functional. The results confirmed our hypothesis that the phospholipid membrane generated at the LC interface could be reused after the enzymatic reaction.

Detection limit of thrombin enzymatic activity

Using the optimized conditions determined above, the detection limit of thrombin enzymatic activity was investigated. When an aqueous solution of 2.5 ng/mL thrombin was introduced onto the PLA-disrupted phospholipid membrane at the aqueous/LC interface; the optical appearance of the LCs changed from bright to dark within 30 min (Fig. 5A), indicating that the LCs underwent an orientational transition from a planar to a homeotropic state after the enzymatic reaction between thrombin and PLA. When we transferred an aqueous solution of 0.25 ng/mL (6.7 pM) thrombin onto the phospholipid membrane disrupted by PLA, the optical texture of 5CB changed from bright to nearly black; only small domains remained bright after 1 h (Fig. 5B). These results demonstrated that the concentration of PLA peptides decreased because of the enzymatic reaction. We also immersed the PLA-disrupted phospholipid membrane into an aqueous solution of 0.025 ng/mL thrombin at the LC interface. The optical response remained bright for 2 h (Fig. 5C), which indicated that the orientation of the LCs remained in a planar state after the enzymatic reaction. This result suggested that enzymatic cleavage by thrombin did not markedly decrease the concentration of PLA peptides. Thus, the concentration of PLA was still sufficient to disrupt the integrity and organization of phospholipids at the LC interface such that the optical response of 5CB remained bright. These results suggested that the detection limit of thrombin in this system was approximately 0.25 ng/mL (6.7 pM).

As an effort to quantify the luminosity of the LC image, we used ImageJ to convert images to gray scale, and the average pixel brightness of a region was calculated. The luminosity of the optical images of 5CB supporting a phospholipid membrane was evaluated after contact with an aqueous solution of 0.032 mg/mL PLA and followed by incubation with thrombin solutions at different concentrations. A linear fit of luminosity was conducted and a basic standard curve was obtained (Fig. 5D).

Herein, we have demonstrated for the first time that an LC-based optical sensor is a highly sensitive and advantageous

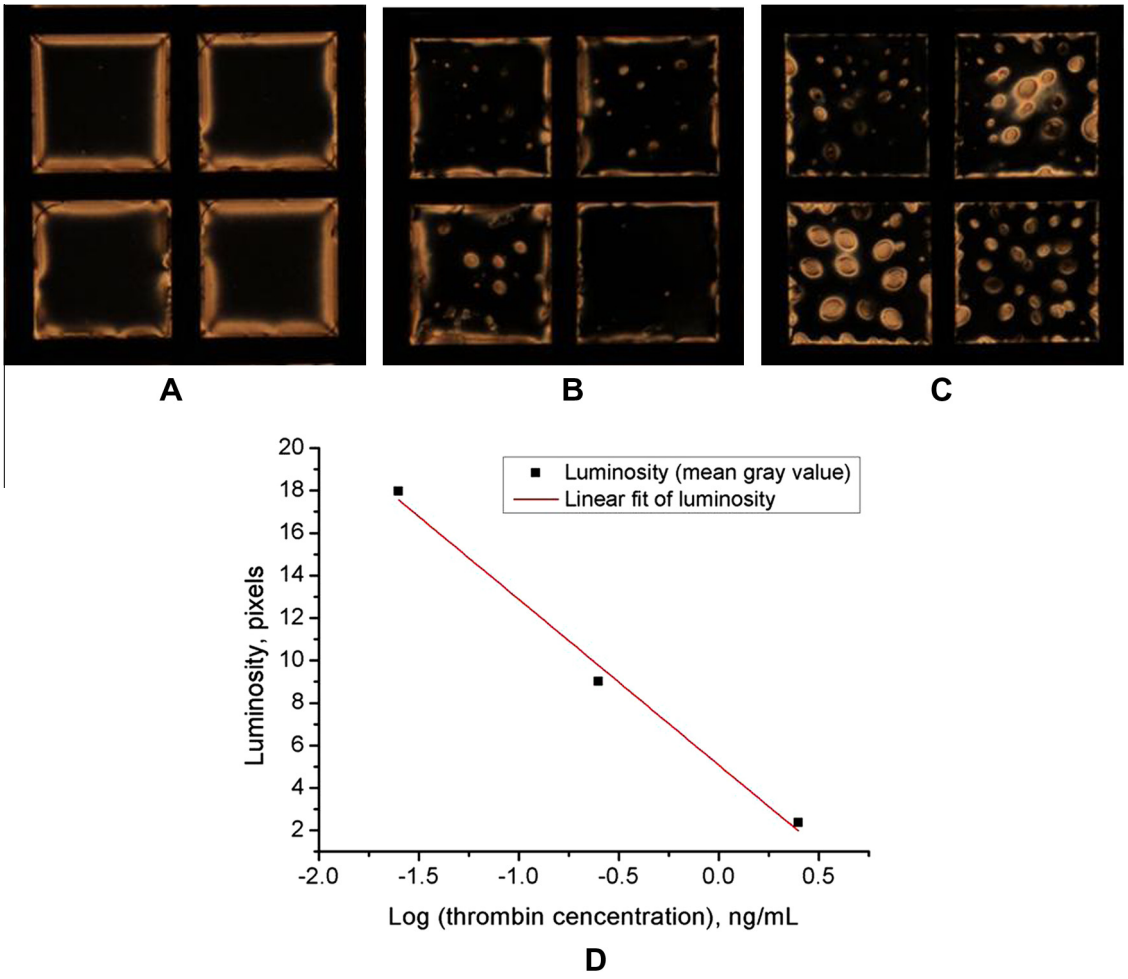

Fig.5. Polarized light microscopy images of LCs supporting a DOPG monolayer at the LC interface in contact with an aqueous solution of 0.032 mg/mL PLA and (A) an aqueous solution of 2.5 ng/mL thrombin for 30 min; (B) an aqueous solution of 0.25 ng/mL thrombin for 1 h; (C) an aqueous solution of 0.025 ng/mL thrombin for 2 h; and (D) luminosity (mean gray value) of LC images as a function of thrombin concentration.

approach for thrombin detection. A correlation between the thrombin concentration and the responsive optical signal was established. The detection limit of the LC-based interface sensor, defined in terms of the thrombin concentration that produced an optical signal transition, was estimated to be 0.25 ng/mL (6.7 pM). This detection limit is 600 times lower than that of a previously reported enzyme-linked aptamer assay (ELAA) for the detection of thrombin [24]. ELAA requires an antibody and aptamer, whereas our LC-based sensor requires no protein labeling, tedious procedures, or complex instrumentation.

The concentration of thrombin in blood varies from the nanomolar to the millimolar range during coagulation [25]. However, the thrombin concentration in blood is lower under normal conditions [26]. Clinically, blood thrombin levels are between 50 and 500 nM during the early stage of thrombosis, but thrombin levels can reach picomolar (pM) concentrations under some conditions [27,28]. Therefore, the sensitive detection of thrombin is important for reliable diagnosis, and a detection limit of 6.7 pM thrombin has diagnostic potential. The improvement of the detection limit can be explained by the increased ratio of enzymes to PLA at the aqueous/LC interface, which amplified the detection response by increasing the number of thrombin molecules per unit interacting with PLA. This simple and quick method for the visualization of thrombin might provide the basis for a rapid detection strategy.

Conclusions

In summary, we have described a highly sensitive and reliable LC-based method for the detection of thrombin. Changes in the orientation of LCs, coupled to the interactions between a polyelectrolyte and a phospholipid monolayer, were used to image enzymatic events at an aqueous/LC interface. The integrity and organization of a negatively charged phospholipid monolayer at the LC interface were disrupted by the addition of a positively charged polyelectrolyte because of electrostatic interactions between the monolayer and the polyelectrolyte. Under a polarized microscope, a dark to bright shift was observed after the introduction of PLA onto the DOPG membrane. Because thrombin catalyzed the hydrolysis of PLA bound to the DOPG monolayer, the electrostatic interactions between PLA and DOPG were weakened or eliminated on addition of the enzyme, and the organization of the phospholipid membrane was restored. Thus, the optical appearance of the LCs reverted from bright to dark, indicating that the orientation of the LCs transitioned from a planar to a homeotropic state. The detection limit of this system was approximately 0.25 ng/mL (6.7 pM) thrombin. We also demonstrated that the phospholipid membrane generated at the LC interface could be reused after the enzymatic reaction. These results indicate that our LC-based sensor offers a highly sensitive, reproducible, and rapid method for the detection of thrombin at clinically relevant concentrations.

Acknowledgments

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