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Label-free optical detection of thrombin using a liquid crystal-based aptasensor

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ABSTRACT

We describe a real-time, label-free aptasensor that utilizes the orientational properties of nematic liquid crystals (LCs) for detection of thrombin. The imaging principle is based on the homeotropic orientation of LC films, which can be influenced by thrombin-specific aptamers and *N*,*N*-dimethyl-N-(3-(trimethoxysilyl)propyl)-1-octadecanaminiuchloride (DMOAP) immobilized on glass slides. The interactions between thrombin and its specific aptamer, including ionic and hydrogen bonds, can interfere with the homeotropic alignment of LCs, leading to changes from 'dark' to 'bright' responses that can be visualized simply using crossed polarizers. We describe the condition for maintaining the homeotropic state of LCs, which are suitable for thrombin-specific aptamer experiments, and reactions between aptamer and several enzymes, including thrombin. This novel simple LC-based aptasensor has not only high specificity, but also high sensitivity (low detection limit of 1 pg/mL (26.7 fM)). We also note the blood coagulation reaction between fibrinogen and thrombin, which confirms that the aptamer-bound thrombin is auto-enzymatic. Our result shows that the sensitivity of the proposed sensor increases (1 pg/mL to 0.1 pg/mL) using fibrinogen.

1. Introduction

Thrombin is the main effector protease of the coagulation cascade in which circulating fibrinogen is converted to fibrin monomers, which then polymerize to form fibrin polymer - the fibrous matrix in blood clots. [1–3] Owing to its hormone-like properties, thrombin is involved in thrombosis and platelet activation. It also plays a central role in a number of cardiovascular diseases. [4,5] Excessive thrombin levels in the body can result in thromboembolic diseases or even death, while thrombin insufficiency can induce excessive bleeding. During the coagulation process, the concentration of thrombin markedly varies, from nM to mM. [6,7] Moreover, monitoring thrombin is of significant importance for early diagnostics of some diseases, such as hemorrhagic and thromboembolic complications, and Alzheimer's disease, while thrombin also serves as a useful marker in the diagnosis of pulmonary metastasis. [8–11] Therefore, it is very important to develop high-sensitivity and high-selectivity sensors for thrombin detection that can be applied not only in research, but also in clinical diagnosis.

Aptamers are DNA or RNA sequences that in vitro were shown to bind to their specific molecular targets. [12–14] Because novel aptamers can be customized, in addition to their generally impressive selectivity and affinity, they have been widely used for recognition in the field of biosensing. [15,16] The affinity of aptamers to their targets can rival that of antibodies, owing to their dissociation constants being as low as in the picomolar (pM) range. [17,18] Unlike antibodies, aptamers can be selected under non-physiological conditions to bind to targets that are either too small or too toxic for antibodies to elicit their immune responses. [19,20] Because aptamers can specifically bind to a wide range of targets, their usage in sensors for in situ and real-time detection and quantification is increasingly considered. Aptamers have been used in a variety of sensing technologies, [21–23] such as fluorescent, [24–29] colorimetric, [30–33] magnetic, [34–36] electrochemical, [37–41] and phase change sensors. [42] Nonetheless, electronic aptamer-based sensors have been described to either require additional exogenous reagents or to be susceptible to interference from contaminants. For example, aptamer-based sensors that utilize the quartz crystal microbalance [43,44] and the surface plasmon resonance, [45,46] although having demonstrated impressive sensitivity and detection speed, are prone to false positives owing to nonspecific binding. In addition, the detection approach based on the aptamer-thrombin interaction has been reported to require complex and multi-step pre-processing, as well as the addition of an exogenous redox label. [47]

Liquid crystalline materials are being increasingly considered in a wide range of sensing and interfacial fields. [48–56] The arrangement/orientation of liquid crystals (LCs) is highly sensitive to interfacial

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molecular events; such events can lead to changes in the optical appearance of the LCs. For example, LCs respond to and amplify small changes in temperature, shear, and electric/magnetic fields of solid surfaces, with which they are in contact. This qualifies LCs as 'molecular magnifying glasses', allowing events that occur on the nanoscale to be observed by a naked eye (and far-field optics), without the need of additional instrumentation. In addition, LC-based methods have reportedly offered potential advantages over conventional techniques because they do not require complex instrumentation or labeling (i.e., enzymatic or fluorescent labeling).

In the present study, a new, label-free, real-time liquid crystal sensor based on a thrombin-specific aptamer is proposed. The study aimed not only at critically evaluating the importance of simple key steps in the aptasensor development that have significant implications on its analytical performance, including selectivity, reproducibility, and stability, but also at studying the effect of the aptamer immobilization procedure (chemistry, length, concentration, and pretreatment of the aptamer), in addition to the binding conditions used in the assay. The activity of thrombin in thrombin-induced fibrin formation was also evaluated. The developed sensor exhibited high specificity toward thrombin over other proteins and enzymes. Using other types of aptamers targeting other proteins, we envision that the proposed approach may hold a great promise as a universal platform for simple, sensitive, and selective detection of other proteins.

2. Experimental section

2.1. Materials

4-Cyano-4′-pentylbiphenyl (5CB) nematic liquid crystals were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Glass microscope slides were purchased from Matsunami Glass Ind., Ltd. (Tokyo, Japan). Ethyl alcohol was acquired from Duksan Pure Chemical Co., Ltd. (Ansan, South Korea). Sulfuric acid and hydrogen peroxide (30% (w/v)) were purchased from Daejung Chemicals & Metals Co., Ltd. (Siheung-si, South Korea). 3-(Trimethoxysilyl)propyl octadecyl dimethyl ammonium chloride (DMOAP), (3-aminopropyl)triethoxysilane (APTES), glutaraldehyde solution (GA), glycine, human plasma thrombin, human plasma fibrinogen, and phosphate buffer saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thrombin aptamer (5′-GGT TGG TGT GGT TGG/3AmMO/−3′) was purchased from Mbiotech, Inc. (Gyeonggi-do, South Korea). All aqueous solutions were prepared with deionized water (DI water; 18.2 MΩ/cm) obtained from a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Surface modification of microscope glass slides with DMOAP

Microscope glass slides were immersed in a piranha solution (a mixture of 30% (*v*/v) hydrogen peroxide and 70% (v/v) sulfuric acid) for 30 min at 80 °C [Warning: a piranha solution reacts violently with organic compounds. The solution should be treated with extreme caution; it should not be stored in closed containers]. The glass slides were rinsed successively with DI water, ethanol, and methanol, and then dried under a stream of gaseous nitrogen. After that, the slides were heated overnight at 120 °C in an oven prior to DMOAP coating. Subsequently, the 'piranha-cleaned' slides were immersed in a 0.35% (v/v) DMOAP aqueous solution for 30 min at room temperature. The DMOAP-coated glass slides were then rinsed 4 times with ethanol followed by DI water.

2.3. Functionalization of microscope glass slides with APTES/DMOAP

The piranha-free glass slides were immersed in an APTES/DMOAP ethanol solution (1% (v/v) APTES and DMOAP) for 2 h at 80 °C. The resultant APTES/DMOAP-functionalized glass slides were rinsed 4 times with ethanol followed by DI water, and then soaked in a 1% (*v*/v) GA aqueous solution at room temperature for 1 h. After that, the slides were cleaned 4 times with DI water and then dried under a stream of nitrogen gas.

2.4. Immobilization of thrombin aptamer/thrombin

The GA/DMOAP glass slides were immersed in a thrombin aptamer solution (50 nM) for 2 h at 37 °C. The resultant thrombin aptamer-coated slides were washed 4 times with DI water, and then dried under a stream of nitrogen gas. Subsequently, the slides were submerged in a glycine solution (80 mM) at room temperature for 1 h, to block any unreacted aldehyde groups of the thrombin aptamer molecules. The slides were rinsed with DI water, and then dried with a small stream of nitrogen gas. After that, the thrombin aptamer-immobilized glass slides were immersed in a thrombin solution. The slides were then cleaned with DI water and dried under a stream of gaseous nitrogen.

2.5. Incubation of a fibrinogen solution

The thrombin-immobilized glass slides were incubated with a fibrinogen solution for 1 h at room temperature to allow the reaction between thrombin and fibrinogen. Thereafter, the slides were rinsed 3 times with DI water and dried under a stream of nitrogen gas.

2.6. Preparation of liquid crystal optical cells

A thrombin fragment-coated surface and a DMOAP-functionalized glass slide were used in the preparation of an optical cell. The two glass pieces had a thin space with a spacer (thickness of approximately 12 μm) at each end, and were held together by binder clips. An isotropic phase liquid crystal, 5CB (at > 35 °C) was loaded into an empty space between the two glass pieces using the capillary force. The cell was then slowly cooled down to room temperature to allow the transition of 5CB from the isotropic phase to the nematic phase.

2.7. Characterization

2.7.1. Polarizing optical microscope

The optical texture of the obtained optical cell was examined using a polarizing optical microscope (POM; ECLIPSE LV100POL; Nikon, Tokyo, Japan). All POM images were obtained using a ×4 objective lens and a digital camera (DS-2Mv; Nikon, Tokyo, Japan) that had a resolution of 1600 × 1200 pixels, a shutter speed of 1/30 s, and a gain of ×1.00.

2.7.2. Atomic force microscopy

Atomic force microscopy (AFM; NanoScope IIIa; Veeco Metrology, Santa Barbara, CA, USA) was used for determining the root-mean-square (RMS) roughness and characterization of surface topography of the thrombin-bound substrate. A silicon wafer was used for minimizing the base roughness. Each AFM image was acquired in a tapping mode with 256 sample points per line at a scan rate of 1.0 Hz.

2.7.3. Ellipsometry

Ellipsometric data with deuterium were obtained using an Ellipsometer (Elli-SE(UV)-FM8; Ellipsotechnology, Suwon, South Korea) equipped with halogen lamps (incidence angle: 70°; wavelength: 380–1100 nm). The data were obtained using a silicon wafer. The optical thickness of the sample was calculated based on the three-phase model (ambient: organic; layer: SiO_2-deposited; substrate) and Sellmeier's equation.