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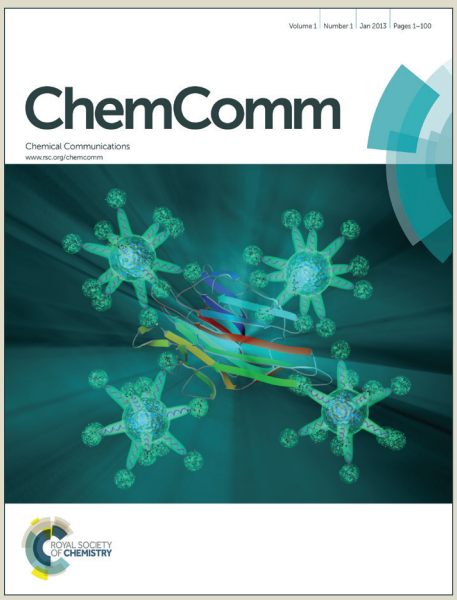



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# Detecting thrombin using a mixture of fluorescent conjugated polyelectrolyte and fibrinogen and its implementation of a logic gate

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**Highly sensitive and selective detection of thrombin is accomplished using emission color-tunable conjugated polyelectrolyte. An implementation of a combined logic gate is realized upon emission modulation of the system including the polymer, fibrinogen, thrombin, and heparin.**

Recently, fluorescent conjugated polymers have been adopted as highly selective and sensitive chemo- and biosensors for the detection of many targets through the well-known signal amplification effect of the conjugated main chain.[1] Conjugated polyelectrolytes have attracted considerable interest for bioapplications[2] and for optoelectronic applications.[3] In bioapplications, the presence of ionic side groups provides favorable electrostatic interaction with biomolecules as well as water-solubility.[4]

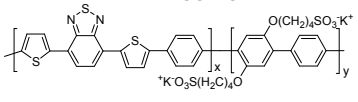
Among the conjugated polyelectrolytes, conjugated polymers with tunable emission colors upon aggregation have gained tremendous attention in sensory platforms for biomolecules.[5] The aggregation-induced exciton migration (energy transfer) in conjugated chains is responsible for the changes in emission colors, usually from a shorter wavelength to a longer one.

Herein, a conjugated polyelectrolyte with such optical properties was employed to detect thrombin. Thrombin plays a crucial role in the coagulation process of blood: it acts as a serine protease to convert soluble fibrinogen to insoluble fibrin clots, and also participate in a catalytic reaction as a coagulation enzyme.[6] This process can be regulated so that clot formation occurs sufficiently rapidly to prevent bleeding from a wound. Thus thrombin is regarded as an important target when investigating suitable anticoagulants and antithrombotics to interfere in blood coagulation.[7]

Much effort has been devoted to replicating the molecular logic gates to perform Boolean logic operations using a variety of molecules and materials that demonstrate various logic outputs as optical or electrical signals in response to special inputs.[8] Among them, fluorometric logic systems are particularly attractive because of their low-cost and ease of readout.[9] To date, several systems have been designed, and some have been implemented practically for multiplexed sensing, intelligent diagnostics, and regulation of protein activity.[10] However, the development of digitalized systems for practical applications using more functionalized materials is still challenging.

In this study, a negatively charged conjugated polyelectrolyte (Scheme 1) was used as a fluorescent probe for thrombin sensing. The polymer has a unique fluorescence property: aggregation-induced blue-to-red fluorescence change. Fibrinogen has a hydrophobic interaction with the negatively charged polyelectrolyte which has a hydrophobic backbone. Because they interacted with each other via weak hydrophobic interaction, no remarkable aggregation was observed, which led to a negligible blue-to-red emission change. By adding thrombin to this mixture, an insoluble fibrin clot was formed from fibrinogen, which could be aggregated with the polyelectrolyte via hydrophobic and electrostatic interactions, which resulted in a blue-to-red color change. Importantly, the specific red emission enhancement of the polyelectrolyte by biomolecules enabled us to use fibrinogen, thrombin, and heparin as inputs to activate combined logic gates and to use the polyelectrolyte as an optical readout signal for logic gate operations. To our knowledge, there have been no reports on sensing protocol for thrombin and build-up of logic gate using emission color-changeable conjugated polyelectrolyte.

The synthetic route to the polyelectrolyte (**P1**) is illustrated in Scheme S1 (ESI).[4d] To realize effective intermolecular energy transfer (blue-to-red change upon aggregation), the molar composition of the polymer should be chosen carefully. The molar composition (x:y) of 0.07:0.93 was employed because only long-wavelength red emission was observed when the value of x exceeded 15 mol% even in a diluted aqueous solution. The bisthienylbenzothiadiazole (TBT) unit, which had more extended conjugation than benzothiadiazole played an important role as an electron acceptor to induce long-wavelength (red) emission via intermolecular energy transfer between polymer chains in the aggregated state.




**P1**

**Scheme 1** Chemical structure of **P1.**

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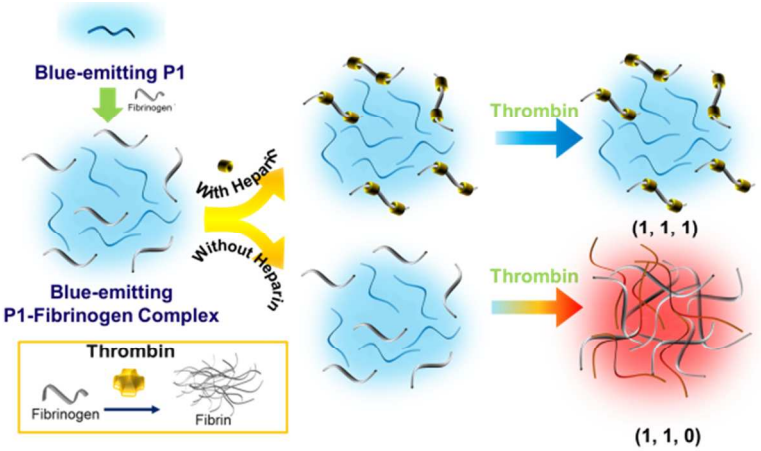
The absorption and emission spectra of **P1** in aqueous solution and in the film are shown in Fig. S1 (ESI). In the fluorescence spectra, the major emissions of **P1** in aqueous solution and in the film are fundamentally different; only a blue emission band at 420 nm that could be assigned to the phenylene units, which are major components in **P1**, was observed in aqueous solution, while a red emission band at 653 nm attributed to TBT units together with the blue one was observed in the solid state. The short-wavelength blue emission of **P1** in aqueous solution resulted from the limited intermolecular energy transfer from phenylene to the TBT unit, while **P1** exhibited a strong red emission in the solid state, which was indicative of enhanced TBT emission via intermolecular exciton migration.[11]




Scheme 2 Schematic illustration of detection of thrombin and application to logic gate in the presence and absence of heparin.

The isoelectric point of fibrinogen is reported to be 5.5; thus, it should have a net negative charge at pH 7.4.[12] Thus, there should be negligible electrostatic interaction between negatively charged **P1** and fibrinogen, which is also net negative at pH 7.4. As a result, fluorescence intensity of **P1** showed little change by the addition of fibrinogen, as shown in Fig. S2 (ESI), implying **P1** did not aggregate in these conditions. Because the fluorescence intensity of **P1** would be changed if more fibrinogen was added (mainly because of the intensified hydrophobic interaction), the amount of fibrinogen should be precisely controlled.

We expected that the fluorescence of the **P1**-fibrinogen (**P1**-F) would change from blue-to-red via the enzymatic activity of thrombin. In the presence of fibrinogen, **P1** did not alter its emission color because of negligible interaction between them. Using the **P1**-F mixture, the thrombin-detection mechanism consists of three steps. 1) A blue emission can be seen in aqueous **P1** solution. 2) The initial blue emission of **P1** is maintained upon addition of fibrinogen to the **P1** solution. 3) When thrombin is added to the mixture, fibrinogen degrades to form water-insoluble fibrin by enzymatic action of the thrombin, in which fibrin has a more favorable interaction with **P1**, forming aggregates of a water-insoluble **P1**-fibrin via electrostatic and hydrophobic interactions. As a result, intermolecular exciton migraion of **P1** occurs readily with a blue-to-red emission change. The concentration of thrombin can be estimated via the emission change of **P1** (bottom flow in Scheme 2). At Stage 1, heparin can be used as a coagulation inhibitor to investigate the enzymatic activity of thrombin on fibrinogen degradation to form a fibrin clot by means of the blue-to-red emission change of **P1** (upper flow in Scheme 2).[14]

The changes in fluorescence spectra of the **P1**–F mixture upon addition of thrombin in 20 mM HEPES buffer at pH 7.4 are shown in Fig. 1a. The emission spectrum of **P1**–F solution is dominated by the short-wavelength blue emission at 420 nm with a weak long wavelength red emission (653 nm). Upon exposure to thrombin, both emissions increase, although the increment in the red emission is greater than that of the blue one (Fig. 1b). It is reported that the increases in both short- and long-wavelength emissions is attributed to the cooperative electrostatic and hydrophobic driving forces between **P1** and fibrin, formed from fibrinogen by the action of the thrombin.[4b,5b] The larger increment in red emission gives a clear fluorescence color change (though relatively smaller intensity than blue). The **P1**-F mixture fluoresces blue, which turns to violet on the addition of thrombin, and, finally, to red with further addition of thrombin (Fig. 1c), which allows naked-eye quantification of thrombin.

To understand its excellent sensing selectivity, the **P1**-F mixture was investigated in the presence of other enzymes, such as pepsin, trypsin, and glucose oxidase (GOx) (Fig. S3, ESI). Comparably smaller increment of fluorescence intensity both at 420 nm and at 653 nm was observed upon addition of other enzymes, which implies negligible interference of other enzymes to thrombin detection and demonstrates the high selectivity of this system (Fig. S4, ESI). The enhancement of long-wavelength emission was not affected by the presence of other enzymes. This negligible interference from other enzymes suggests that the **P1**-F system has significant selectivity toward thrombin.

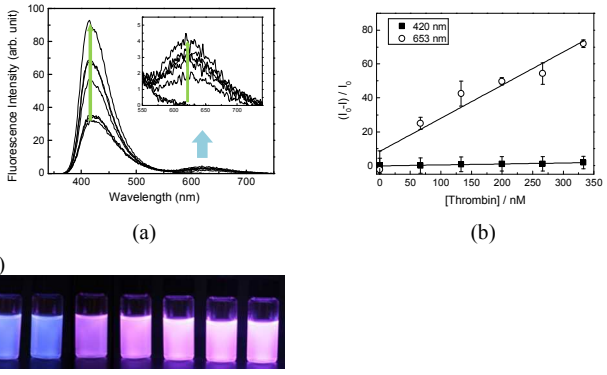



**Fig. 1** (a), (b) Changes in emission intensity of **P1**-F mixture by addition of thrombin with various concentrations and (c) photos of **P1**-F in the presence of thrombin in 20 mM HEPES buffer at pH 7.4. Incubation time 45 min. Excitation wavelength, $\lambda_{ex}$ = 330 nm; [**P1**] = 1.0 x $10^{-4}$ M; [fibrinogen] = 1.0 x $10^{-7}$ M; [thrombin] = 0, 6.66 x $10^{-8}$, 1.33 x $10^{-7}$, 1.33 x $10^{-7}$, 2.00 x $10^{-7}$, 2.66 x $10^{-7}$, 3.30 x $10^{-7}$ M (bottom to top for (a) and left to right for (c)). $I_o$ and I correspond to the emission intensities in the absence and presence of thrombin, respectively.

This phenomenon is based on the intermolecular aggregation of **P1** with fibrin (formed from fibrinogen by the hydrolytic action of thrombin). It should be emphasized that the formation of fibrin is essential for intermolecular aggregation with **P1** via electrostatic as well as hydrophobic interactions,[7c] which leads to a fluorescence color change. Because fibrinogen plays a key

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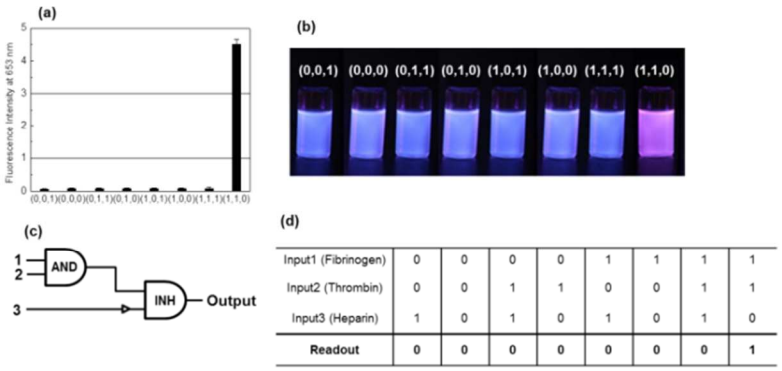
role as a captor for **P1** to form a mixture and as a producer to be a water-insoluble fibrin (Fig. S5, ESI), the optimal amount of **P1**-F mixture should be estimated. The most sensitive detection of thrombin can be achieved when using fibrinogen of about ~$10^{-7}$ M. The limit of detection for thrombin was estimated to be 0.175x$10^{-9}$ M, which is comparabe with previous results from other sensing methods.[6d,7c]

To ensure the enzymatic activity of thrombin in the **P1**-F system, the influence of inhibitor was investigated using heparin, which is known to be a coagulation inhibitor for thrombin. Thrombin was added to **P1**-F mixture in the presence of heparin. In this case, thrombin could not hydrolyze fibrinogen to produce fibrin because of the inhibition of heparin. Thereby, the fluorescence color becomes similar to the initial emission color of the **P1**-F mixture and, ultimately, the initial blue emission is almost recovered (Fig. S6, ESI). This clearly indicates that heparin plays a crucial role in inhibiting the formation of fibrin and thus prevents aggregation of **P1**.

Finally, these results can be interpreted as an implementation of a combined logic operation.[13] The characteristic color changes in the presence of four inputs (**P1**, fibrinogen, thrombin, and heparin) enable us to provide more complicated logic circuits. For input, 1 was defined as the presence of four species and 0 was defined as their absence. As shown in Fig. 2, a logic gate composed of AND and INH gates was realized when the three inputs were used as input signals for the system. The output signals are defined as 1 and 0 when the solution of **P1** emits red (the ratio of fluorescence intensity at 653 nm is above 3) and blue (below 1), respectively. An AND gate is represented by the situation in which the output of the gate is 1 if both inputs are 1, which means both fibrinogen and thrombin are prerequisite to obtain an output signal 1 (red emission). An INH gate is a combination gates of AND and NOT gates. Thus, only in the presence of fibrinogen and thrombin as well as absence of heparin, i.e., an input of (1,1,0), a significant increase in red fluorescence can be observed at 653 nm (output 1), suggesting that the system worked as a combined AND-INH logic gate. These results indicate that a combined logic system of AND-INH logic operation is successfully accomplished based on the water-soluble conjugated polymer-fibrinogen mixture in the presence of fibrinogen and heparin.

In conclusion, a conjugated polyelectrolyte was prepared to investigate its utility in the efficient detection of thrombin via emission color change. The unique ability of **P1** to form a mixture with fibrinogen led to the accomplishment of the fibrin-based thrombin-sensing material. Based on this system, a combined logic gate operation of AND-INH was designed and realized with various combinations of the external stimuli including **P1**, fibrinogen, thrombin, and heparin. Financial support from the National Research Foundation (NRF) of Korean government through Basic Science Research Program (2012R1A2A2A01004979) is gratefully acknowledged.




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Input1 (Fibrinogen) | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| Input2 (Thrombin) | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| Input3 (Heparin) | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| **Readout** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** |

**Fig. 2** (a) Relative fluorescence intensities at 653 nm for the AND-INH logic gate, (b) photograph of **P1** with three inputs under UV light (365 nm); [**P1**] = 1.0 x $10^{-4}$ M; [fibrinogen] = 1.0 x $10^{-7}$ M; [thrombin] = 3.30 x $10^{-7}$ M; [heparin] = 3.30 x $10^{-7}$ M, (c) Circuit for the 3-input logic scheme, and (d) truth table for the AND-INH gate.

† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [Synthesis and optical properties of the polymer, variation in fluorescence properties]. See DOI: 10.1039/c000000x/

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