Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

A Sensitive and Reliable Detection of Thrombin via Enzyme-Precipitate-Coating-Linked Aptamer Assay

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I: Experimental Section

Chemicals and Materials Anti-thrombin aptamer 1 (Tapt1, 15 bases, 5'-NH_2-6C-GGT TGG TGT GGT TGG-3') and anti-thrombin aptamer 2 (Tapt2, 29 bases, 5'- NH_2-6C-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3') were purchased from Geno-Tech Co (Dae-jeon, Korea). Carboxylated magnetic beads (MBs, 1 μm diameter) were purchased from Invitrogen (Carlsbad, CA, USA), and multi-walled carbon nanotubes (MWCNTs, 30 ± 15 nm in outer diameter and 1~5 μm in length, purity > 95%) were purchased from Nanolab, Inc. (Newton, MA, USA). N-ethyl-N'-(dimethlaminopropyl) carbodiimide hydrochloride (EDC) and 2-[N-morpholino]ethanesulfonicacid buffer (MES buffer, 0.1 M, pH 6.5) were purchased from Pierce (Rockford, IL, USA). N-hydroxysuccinimde (NHS) solution was purchased from Alfa Aesar (Ward Hill, MA, USA). Glucose oxidase (GOx), thrombin, phosphate buffered saline (PBS, 10 mM, pH 7.4), glutaraldehyde (GA), ammonium sulfate (65% $(NH_4)_2SO_4$ solution), bovine serum albumin (BSA), Tween-20, D-glucose and H_2SO_4 (98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). HNO_3 (70%) was purchased from Daejung Chemicals & Metals Co (Shiheung City, Korea).

Conjugation of anti-thrombin aptamer 1 (Tapt1) with magnetic beads Carboxylated MBs (0.1 mg, 1.0 x 10^8 MBs/mg) were briefly washed with 1 mL of 0.01 M NaOH, and treated with 1 mL of 106 mM EDC at 4ºC under shaking (200 rpm) for 30 min. After washing with 1 mL of PBS (10 mM, pH 7.4), EDC-activated MBs were incubated in 1 mL of PBS (10 mM, pH 7.4) containing 2 μM Tapt1 at room temperature under shaking (200 rpm) for 1 h. After excessive washing with 1 mL of PBS (10 mM, pH 7.4), unreacted EDC-activated groups on the MB surface were capped by incubating the sample in 1 mL of capping solution (10 mM PBS containing 0.1% BSA) at room temperature under shaking (200 rpm) for 1 h. Tapt1 conjugated MBs (MB-Tapt1) were washed three times with 1 mL of PBS (10 mM, pH 7.4), re-suspended in 1 mL of PBS (10 mM, pH 7.4), and stored at 4ºC until use.

Preparation of EPC-GOx/CNTs and Tapt2-conjugated EPC-GOx/CNTs EPC-GOx/CNTs were prepared by following the protocol of Kim et al.[1] CNTs were treated with acids (7.5 mL of 98% H_2SO_4 and 2.5 mL of 70% HNO_3), and acid-treated CNTs were incubated in 1 mL of 5.32 mM EDC and 86.8 mM NHS at room temperature under shaking (200 rpm) for 1 h. EDC-activated CNTs were mixed with the GOx solution at room temperature under shaking (100 rpm) for 1 h. Ammonium sulfate was added, and the mixture was shaken at 100 rpm for 30 min. Finally, 25% GA solution was added in the final concentration of 1.2 mM GA, and the mixture was incubated at 4°C under shaking (50 rpm) for overnight. The resulting EPC-GOx/CNTs were washed five times with sodium phosphate buffer (PB buffer, 0.1 M, pH 7.4), and washed with DI water.

For the conjugation with Tapt2, EPC-GOx/CNTs were mixed with 1 mL of EDC solution (106 mM) and incubated at room temperature under shaking (200 rpm) for 30 min. After washing with DI water, EDC-activated EPC-GOx/CNTs were re-suspended in 1 mL of 2.5 μM Tapt2 PBS (10 mM, pH 7.4), and incubated at room temperature under shaking (200 rpm) for 1 h. Unbound aptamers were removed by decanting the supernatant after centrifugation at 1300 rpm for 10 min, and Tapt2-conjugated EPC-GOx/CNTs (EPC-Tapt2) were washed with PBS (10 mM, pH 7.4). After treated with the capping solution (10 mM PBS containing 0.1% BSA), EPC-Tapt2 were re-suspended in PBS (10 mM, pH 7.4), and stored at 4ºC until use.

EPC-linked aptamer assay (EPC-LAA) for the thrombin detection MB-Tapt1 (0.1 mg MBs, 1.0 x 10^8 MBs) were dispersed in 1 mL of PBS (10 mM, pH 7.4) containing various concentrations of thrombin (0 ng/mL ~ 1 μg/mL). For the capture of thrombin by MB-Tapt1, the mixture was incubated at room temperature under shaking (200 rpm) for 1 h. Thrombin-bound MB-Tapt1 were collected by using a magnet, and washed five times with 1 mL of PBS (10 mM, pH 7.4) and three times with 1 mL of washing buffer (10 mM PBS containing 0.05% Tween 20). After re-suspending thrombin-bound MB-Tapt1 in 1 mL of PBS (10 mM, pH 7.4), 1 mL of EPC-Tapt2 suspension was added, and the mixture was incubated at room temperature under shaking (200 rpm) for 1 h. The complex was washed three times with 1 mL of wash buffer (10 mM PBS containing 0.05% Tween 20), and then was resuspended 1 mL of PBS (10 mM, pH 7.4). The sandwich complex of MBs and EPC-GOx/CNTs (MB/Thrombin/EPC), mediated by thrombin, was used for the GOx assay[2] that allows for the quantitative analysis of thrombin concentration in the sample. In more detail, 10 μL of MB/Thrombin/EPC was mixed with 990 μL of the reaction cocktail containing 93.78 mM glucose, 0.17 mM o-dianisidine and 0.06 units/mL horseradish peroxidase, and the mixture was incubated at room temperature. GOx-catalyzed glucose oxidation generated hydrogen peroxide, which was used in peroxidase-catalyzed oxidation of o-dianisidine. The time-dependent increase of absorbance at 500 nm, due to the oxidation of o-dianisidine, was measured by using a spectrophotometer (Shimadzu, UV-1800), and used for the calculation of initial rate as an enzyme activity of each sample's sandwich complex. All activity measurements were done at least in two independent assays.

Performance stability of EPC-LAA To investigate the performance stability of EPC-LAA, the preparations of MB-Tapt1 and EPC-Tapt2 were incubated at 40°C. The aliquots of MB-Tapt1 and EPC-Tapt2 were removed from each stock time-dependently, and used for the detection of thrombin (100 ng/mL) at room temperature. At each time point, EPC-LAA was performed by measuring the GOx activity of the sandwiched MB/Thrombin/EPC complex. The relative activity, defined by the ratio of residual activity at each time point to the initial activity, was used to check the performance stability of EPC-LAA after incubation at 40°C. As a control, GOx was covalently attached onto CNTs (CA-GOx/CNTs),[1] and Tapt2 was

conjugated onto CA-GOx/CNTs (CA-Tapt2). Then, CA-Tapt2 was also incubated at 40°C, and the CA-LAA was performed in the same way.

II. Supporting Figures and Discussions

Quantification of conjugated aptamer and captured thrombin For the conjugation of Tapt1 on MBs, EDC-activated MBs were incubated in 1 mL of PBS (10 mM, pH 7.4) containing 2 μM Tapt1 at room temperature under shaking (200 rpm) for 1 h. The amount of conjugated Tapt1 with MBs was estimated by the difference of absorbance at 260 nm in solution before and after the conjugation. As a result, 4.69 μg of Tapt1 was conjugated onto MBs (0.1 mg MBs; 1.0×10^8 MBs).

Prior to the EPC-LAA, the capacity of MB-Tapt1 (4.69 μg Tapt1 on 0.1 mg MBs, 1.0×10^8 MB-Tapt1) for the thrombin detection was tested. MB-Tapt1 were dispersed in 1 mL of PBS (10 mM, pH 7.4) containing excessive concentrations of thrombin (10 μg/mL). For the capture of thrombin by MB-Tapt1, the mixture was incubated at room temperature under shaking (200 rpm) for 1 h. Thrombin-bound MB-Tapt1 were captured by using a magnet, and any unbound thrombin was collected in another tube. The thrombin concentration was analyzed using bicinchoninic acid (BCA) assay, and the amount of bound thrombin was calculated from the difference of thrombin concentration before and after thrombin binding. The amount of bound thrombin was estimated to be 1.25 μg thrombin per 0.1 mg MB-Tapt1. Therefore, 0.1 mg of MB-Tapt1 would be good enough to capture more than 1 μg of thrombin. It is anticipated that the saturation level would be observed in the presence of 1.25 ug thrombin. On the other hand, the saturation level and dynamic range can be potentially controlled by the amount of MBs to be used for EPC-LAA.

EDC-activated EPC-GOx/CNTs were re-suspended in 1 mL of 2.5 μM Tapt2 in PBS (10 mM, pH 7.4), and incubated at room temperature under shaking (200 rpm) for 1 h. The supernatant was collected after centrifugation at 1300 rpm for 10 min. The amount of conjugated Tapt2 with EPC-GOx/CNTs was estimated to be 9.61 μg of Tapt2 was conjugated onto EPC-GOx/CNTs (1.0 mg CNTs) from the difference of absorbance at 260 nm in solution before and after the conjugation.

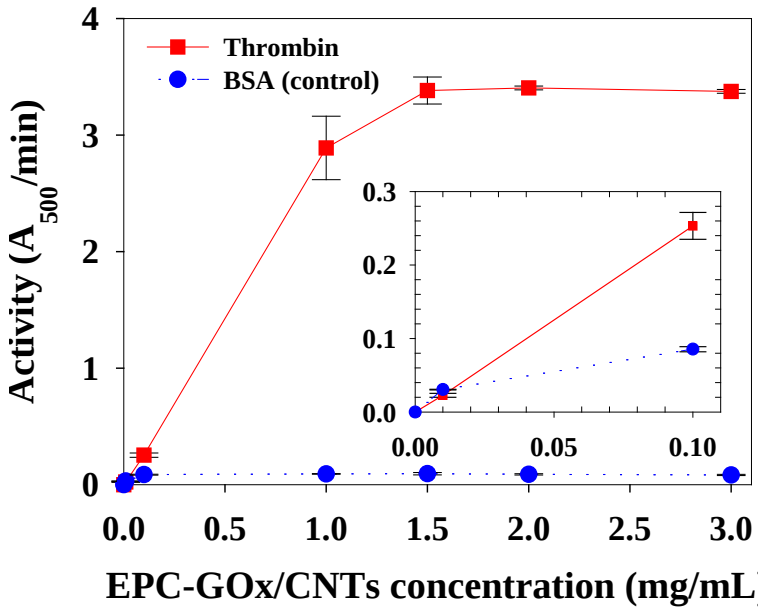

Figure S1. Optimization for the concentration of EPC-GOx/CNTs in the protocol of EPC-LAA. Bovine serum albumin (BSA) was used for the control experiment.

To optimize the protocol of EPC-LAA, various concentrations of EPC-Tapt2 were tested for the quantitative detection of thrombin. The spectroscopic signal (increase at the absorbance of 500 nm) could be obtained by measuring the GOx activity via the peroxidase-catalyzed oxidation of *o*-dianisidine using hydrogen peroxide, which is generated from GOx-catalyzed oxidation of glucose. The detection of thrombin (1 μg/mL) was performed with EPC-Tapt2 in various concentrations (0 - 3.0 mg/mL CNTs) and an excessive concentration of MB-Tapt1 (0.1 mg/mL MBs). Due to the difficulties in measuring the exact amounts of MB-Tapt1 and EPC-Tapt2, the concentrations of MBs and CNTs were used to represent the concentrations of MB-Tapt1 and EPC-Tapt2, respectively. The spectroscopic signal in the presence of 1 μg/mL thrombin increased as the concentration of EPC-GOx/CNTs was increased up to 1.5 mg/mL CNTs (EPC-Tapt2), and the further increase of EPC-Tapt2 resulted in the signal saturation. The presence of 1 μg/mL BSA with no thrombin showed negligible signals.

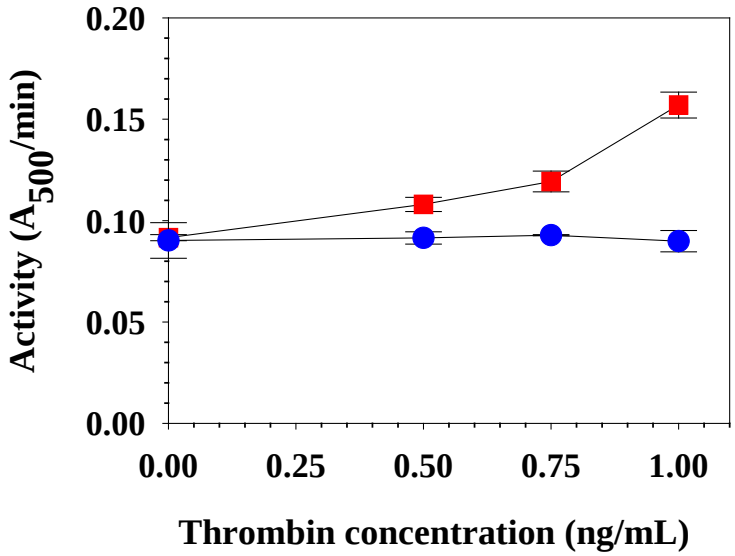

Figure S2. Blow-up inset figure of Figure 3 for the estimation of LOD. Based on triplet set of data with six measurements, the blank signal was estimated to be 0.0916 ± 0.0015. The sum of averaged blank signal and three times their standard deviation was calculated to be 0.0961. The signal in the presence of 0.5 ng/mL thrombin was 0.1080 ± 0.0035, which represents a greater signal than the above sum (blank signal + 3 x standard deviation of blank signal = 0.0961). In this way, the LOD of EPC-LAA was estimated to be 0.5 ng/mL thrombin.

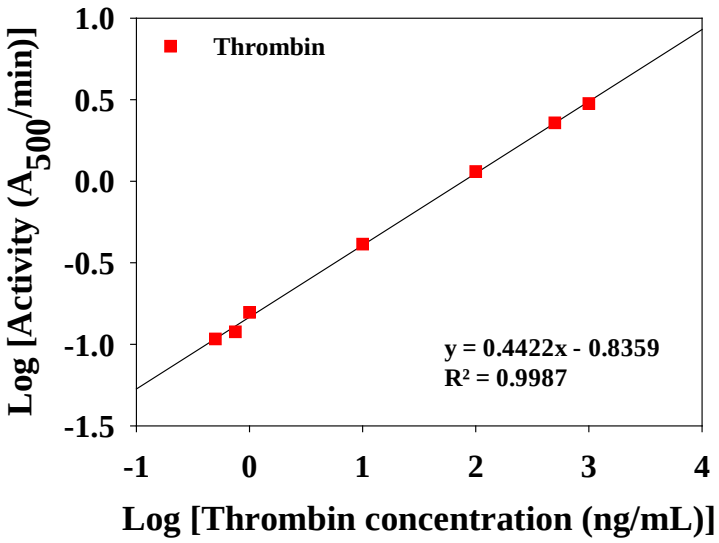

Figure S3. Hill's correlation between the initial thrombin concentration and the GOx activity of sandwich complexes.

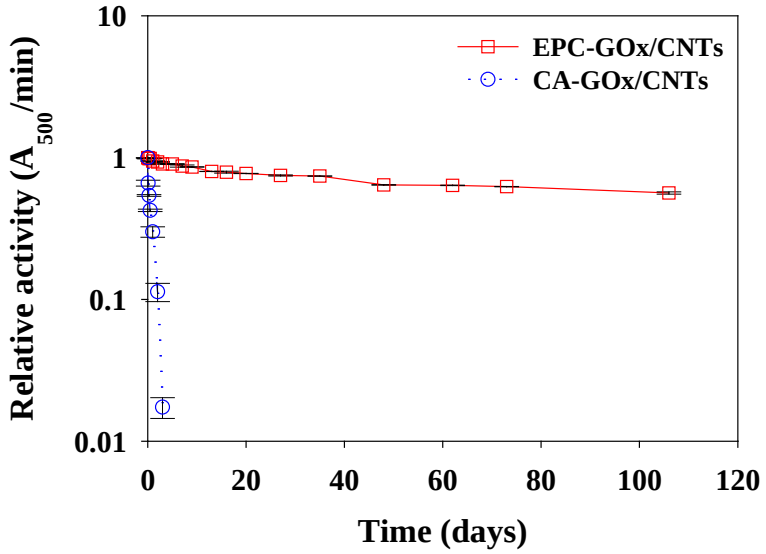

Figure S4. Stabilities of EPC-GOx/CNTs and covalently-attached GOx/CNTs (CA-GOx/CNTs) at 40°C. The relative activity represents the ratio of residual activity at each time point to the initial activity of each sample.

One of the significant advantages in using EPC-GOx/CNTs can be found from their good stability under harsh condition for a long period of time.[1] As a control, covalently-attached GOx on CNTs (CA-GOx/CNTs) were prepared, and the stabilities were compared by checking the residual activity after incubation at 40°C. EPC-GOx/CNTs maintained about 80% of initial activity after 13-days incubation at 40°C while CA-GOx/CNTs showed less than 2% of initial activity even after 3-days.

References

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2. H. U. Bergmeyer, K. Gawehn and M. Grassl, in *in Methods of Enzymatic Analysis*, ed. H. U. Bergmeyer, Academic Press Inc., New York, NY, 1974, pp. 457-458.