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Magnetic force assisted electrochemical sensor for the detection of thrombin with aptamer-antibody sandwich formation

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ABSTRACT

A magnetic force assisted electrochemical aptamer-antibody sandwich assay (MESA) was developed for the detection of thrombin as a model protein in serum samples. The MESA using the formation of sandwich complexes on the electrochemical sensor probe for reaction and the removal of unbound bioconjugates from the sensor surface without washing are controlled by a magnetic field. Thrombin was determined by the cathodic currents of a toluidine blue O (TBO) attached with thrombin antibody modified magnetic nanoparticle (MNP) at the sensor surface. To detect thrombin in a serum sample, we applied a thrombin-specific aptamer as the capture molecule bound to the functionalized conducting polymer layer (poly-(2,2′:5′,5″-terthiophene-3′-p-benzoic acid) (pTBA)), and streptavidin and starch coated-MNP was conjugated with biotinylated thrombin antibodies (Ab) and TBO as the bioconjugate (MNP@Ab-TBO). The characterization of MNP@Ab-TBO and sensor probe was performed using voltammetry, impedance spectroscopy, XPS, and UV–VIS spectroscopy. The experimental conditions were optimized in terms of pH, binding time, removal time of unbound bioconjugates, and applied potential. The dynamic ranges of thrombin were from 1.0 to 500 nM with detection limit of 0.49 (± 0.06) nM. The recovery test demonstrates the reliability of the proposed sensing system for a handheld device.

1. Introduction

Thrombin is a serine protease, which plays an important role in the blood coagulation cascade. It catalyzes many coagulation-related reactions including the conversion of fibrinogen to insoluble fibrin (Tasset et al., 1997). The coagulation protease thrombin also regulates critical cellular responses in hemostasis and thrombosis, inflammation, and blood vessel development (Coughlin, 2000). Besides, evidences have been reported to support the hypothesis that thrombin serves to activate tumor growth, metastasis, and angiogenesis (Inuyama et al., 1997; Nierodzik and Karpatkin, 2006). Therefore, it is reasonable to say that thrombin in blood is a relevant marker to estimate some diseases including thrombosis, vascular injuries, and metastatic cancers. Hence, various assay methods including clotting assays (Macfarlane and Biggs, 1953), surface enhanced Raman spectroscopy (Gao et al., 2015a), enzymatic activity assays (Luddington and Baglin, 2004), and immunoassays (Shuman and Majerus, 1976; Rand et al., 1996) have been applied to quantitatively detect thrombin in the blood. Although these methods can provide good performance, they suffer from somewhat drawbacks, such as long analysis time, requiring specialists, and high cost, which make them less suitable for hand-held device applications. Thus, a simple and robust protein detection method is needed. One of candidates is electrochemical sensors using aptamers (Willner and Zayats, 2007; Min et al., 2010) as an alternative thrombin analysis method. This can offer clear advantages of the possibility to miniaturize a detection system with high sensitivity, low cost, independence from sample volume, and less interferences in real samples (Chandra et al., 2012). Hence, there have been much efforts to develop robust electrochemical sensors for the thrombin detection (Gao et al., 2015b, 2016; Qian et al., 2015; Wang et al., 2017).

For the last two decades, aptamers have attracted much attention of researchers in biosensor applications, due to their remarkable features in terms of high specificity and affinity to a wide range of molecular targets, ease of synthesis, high stability, flexibility in labeling at a desired site without loss of activity, and weak immunogenicity (Iqbal et al., 2000; Tan et al., 2004). One of them, thrombin aptamers have been extensively sought and the most widely used to demonstrate the proof-of-concept of the aptamer-based assays or sensors. To do this, aptamers could be immobilized using supporting materials, such as conducting polymers (Moon et al., 2018), gold-thiol interactions (Min et al., 2010), hydrogel particles (Srinivas et al., 2011) or magnetic beads (Tennico et al., 2010), to capture target molecules in sample

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solutions. Of these, conducting polymers having functional group of carboxylic acid or amine are rather excellent substrate than the others to immobilize biomaterial and catalysts on the sensor probe, which have electrically conductive and biologically compatible properties (Koh et al., 2011). Thus, to enhance the sensor performance along with stability and sensitivity, using a functionalized conducting polymer is one of the finest methods to bind biomaterial through the covalent bond formation (Naveen et al., 2017).

Among the electrochemical aptasensor, the sandwich assay format has still been widely applied for the detection of proteins. In case of the target protein having two binding sites for two distinct aptamers, the target can be captured by binding to the capture aptamer on the electrode, and then form a sandwich complex with the second aptamer conjugated with a redox indicator (horseradish peroxidase (Mir et al., 2006), platinum nanoparticles (Polsky et al., 2006), and toluidine blue O (Moon et al., 2017)), which generates electrochemical response. However, it has been reported that only few kinds of proteins have two aptamer binding sites, thus most of the sandwich protein assays have focused on conventional antibody-based immunoassays or assays based on aptamer-antibody pair (He et al., 2007; Zhu et al., 2013). Moreover, the sandwich protein assays still face challenges that they necessarily require time- and reagent-consuming washing and repetitive steps, regardless of the detection methods. For the last decade, the miniaturized point-of-care devices have been studied to provide the advantages of portability and automation using fluorescence sandwich immunoassay (Fan et al., 2008), and ELISA (Chin et al., 2011). However, those devices still require pre-stored buffers and reagents for washing and reaction, as well as external pumping elements to transfer the sample and reagents inside the device. To solve this limitation, a few attempts have been made to eliminate pumping and buffer exchange steps in the sandwich protein assay protocols using electrokinetic mechanisms (Hwang et al., 2010). Nevertheless, this method also has a limitation which efficiency is sensitive to the electrical properties of samples.

To overcome the disadvantage using extra washing step of immunoassay, we introduce a methodology called magnetic force-assisted electrochemical sandwich assay (MESA), where the formation of sandwich complexes on electrochemical sensor probe for reaction and the removal of unbound bioconjugates from the sensor surface are controlled by a magnetic field, and the amount of analyte is quantified by measuring the electrochemical signals from the bioconjugates bound onto the sensor surface. In particular, the electrochemical sandwich assay combined with the magnetic beads have been developed as a purpose of electrode probe (Centi et al., 2007; Eguílaz et al., 2010; Han et al., 2016). However, there were no report using bioconjugated magnetic bead as an electrochemical label to minimize the reaction/washing step, to date.

In this study, to detect thrombin from a serum sample, we applied thrombin-specific aptamer as the capture molecule bound to the functionalized conducting polymer layer (poly-(2,2′:5′,5″-terthiophene-3′-p-benzoic acid) (pTBA)), and streptavidin-starch modified magnetic nanoparticle (MNP) combined with toluidine blue O (TBO) and biotinylated thrombin antibodies (Ab) as the bioconjugates. The MESA enables to quantify the amount of target analytes in a tiny amount of sample droplet without any moving elements or buffer exchange steps as well as without any bulky and expensive detection components. This method would provide a solution for the needs of point-of-care testing market, which have sought a method for simple, automated, rapid, and accurate detection of disease markers from a drop of biological fluids using a handheld device.

2. Experimental

2.1. Materials

Di(propylene glycol) methyl ether, tri(propylene glycol) methyl ether, 1,1′-carbonyldiimidazole (CDI), toluidine blue O (TBO), thrombin, 1-(3-dimethylaminopropyl) −3- ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 100 nm size starch-coated bionized nanoferrite particles with streptavidin on surface (MNP) was purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). Biotin-conjugated human thrombin antibody was purchased from Abcam (Cambridge, UK). The amine terminated thrombin aptamers were obtained from Bioneer Co. (South Korea), which have the following sequences: 5′GGT TGG TGT GGT TGG-C_6 3′amine. Prior to using the aptamer, a stock solution was prepared as follows: the aptamer was denatured for 3 min at 95 °C, incubated for 1 h at 4 °C. A terthiophene monomer bearing a benzoic acid group, 2,2′:5′,5″-terthiophene-3′-p-benzoic acid (TBA) was synthesized according to a previous report (Kim et al., 2012). Phosphate buffered saline (PBS) solutions were prepared with 0.1 M disodium hydrogen phosphate (Sigma-Aldrich), 0.1 M sodium dihydrogen phosphate (Sigma-Aldrich). All the aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q water-purification system (18 MΩ cm).

2.2. Instruments

The screen-printed carbon electrodes (SPCEs) were composed of carbon, Ag/AgCl, and carbon as the working, reference, and counter electrodes, respectively. Carbon and silver inks (Jujo Chemical, Japan) were printed on the polystyrene-based film using a screen printer (BANDO Industrial, Korea). Cyclic voltammogram and chronoamperogram were recorded using a potentiostat (Kosentech Model PT-1). The electrochemical impedance spectra (EIS) were measured with an EG & G Princeton Applied Research PARSTAT 2263 at an open-circuit voltage from 100 kHz down to 100 mHz, at a sampling rate of five points per decade (AC amplitude: 10 mV). The electric-magnet was obtained by BBB incorporation (South Korea). The electric-magnet was operated by Laboratory DC power supply gps 3303 (12 V). The morphology of MNP@Ab-TBO particles was characterized by transmission electron microscopy (TEM; TALOS F200X) and UV–Vis spectrometer (UV-1800).

2.3. Preparation of magnetic nanoparticle conjugate

At first, 5 mL of streptavidin-starch coated magnetic nanoparticles (0.5 mg/mL in 0.02% sodium azide) (MNPs) was incubated with the 0.5 mL of 150 μg/mL biotinylated anti-thrombin antibody for 1 h at room temperature. Thereafter, the MNPs conjugated with antibodies were collected with a permanent magnet, followed by washing with 0.1 M PBS solution. The collected MNPs were incubated in 5 mL of 20 mM CDI in 0.1 M PBS for 6 h at 8 °C. The MNPs were collected by a permanent magnet, then reacted in 5 mM TBO in 0.1 M PBS solution overnight at 8 °C. The MNPs@Ab coated with TBO (MNPs@Ab-TBO) were collected and washed using a permanent magnet.

2.4. Fabrication sensor probes

The screen-printed carbon electrodes (SPCEs) were manufactured on the polyethylene-based film using a screen printer (BANDO industrial, Korea). Silver was coated on the film as conductor, and then carbon was printed as working and counter electrodes. Then insulator was also covered on the top of the film. To modify the surface of the working electrode, 1 mM TBA in a 1:1 mixture of dipropylene glycol methyl ether and tripropylene glycol methyl ether was dropped onto the working electrode, then dried at 60 °C for 1 h in the dry oven. The monomers were electrically polymerized via CV with potential cycling from 0.0 V to 1.2 V (vs. Ag/AgCl) for 2 cycles at 0.1 V/s in 0.1 M PBS (pH 7.4). The amine terminated thrombin aptamer (2 μM) was immobilized on the benzoic acid functionalized conducting polymer layer (poly-TBA) with EDC/NHS coupling. Each modification step was verified using CV and EIS. The sensor chamber was fabricated using the screen-printed electrode, polyethylene cover (thickness: 1.2 mm), and