Biosensors and Bioelectronics 73 (2015) 174–180

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Dual aptamer-immobilized surfaces for improved affinity through multiple target binding in potentiometric thrombin biosensing

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ARTICLE INFO

Article history:
Received 22 April 2015
Received in revised form
25 May 2015
Accepted 29 May 2015
Available online 2 June 2015

Keywords:
DNA aptamer
Thrombin
Field-effect transistors
Self-assembled monolayers
Cyclic voltammetry
Chronocoulometry

ABSTRACT

We developed a label-free and reagent-less potentiometric biosensor with improved affinity for thrombin. Two different oligomeric DNA aptamers that can recognize different epitopes in thrombin were introduced in parallel or serial manners on the sensing surface to capture the target via multiple contacts as found in many biological systems. The spacer and linker in the aptamer probes were optimized for exerting the best performance in molecular recognition. To gain the specificity of the sensor to the target, an antifouling molecule, sulfobeaine-3-undecanethiol (SB), was introduced on the sensor to form a self-assembled monolayer (SAM). Surface characterization revealed that the aptamer probe density was comparable to the distance of the two epitopes in thrombin, while the backfilling SB SAM was tightly aligned on the surface to resist nonspecific adsorption. The apparent binding parameters were obtained by thrombin sensing in potentiometry using the 1:1 Langmuir adsorption model, showing the improved dissociation constants (K_d) with the limit of detection of 5.5 nM on the dual aptamer-immobilized surfaces compared with single aptamer-immobilized ones. A fine control of spacer and linker length in the aptamer ligand was essential to realize the multivalent binding of thrombin on the sensor surface. The findings reported herein are effective for improving the sensitivity of potentiometric biosensor in an affordable way towards detection of tiny amount of biomolecules.

1. Introduction

Label-free electrical biosensing using a field-effect transistor (FET) and ion-sensitive FET (ISFET) is a powerful technique for detecting biomolecules and biochemical reactions in amperometric, potentiometric, and capacitive formats (Bergveld, 2003; Goda et al., 2015; Lee et al., 2009; Matsumoto et al., 2009; Palazzo et al., 2015; Poghossian et al., 2005; Schoning and Poghossian, 2006). The fabrication of FET biosensor is compatible to the complementary metal oxide semiconductor (CMOS) process (Graham et al., 2011) so that it is straightforward to downsizing, high integration, and low-cost production for providing a new modality for home healthcare and decentralized diagnosis by realizing miniaturized and easy-to-operate type of devices. A key issue for measuring biomolecules underlies the development of functional bio-interface which shows a high affinity and selectivity to an analyte on the gate surface in contact with a biological sample. So far, a range of ligands and receptors from natural and synthetic origins has been employed on the gate electrode to show an affinity to a target molecule. The recognition event should occur within the electrical double layer at the electrode–solution interface since the transducing mechanism is based on the field effect, namely the electrostatic interaction of carriers in the semiconductor (i.e., electrons or holes) with innate charges of biomolecule as an analyte. Charges of target molecule located outside the double layer are screened by mobile counterions in an electrolyte solution. The characteristic screening length (i.e., the solution Debye length) results in less than one nanometers under physiological ionic strengths, meaning that an antibody as a general ligand in immunoassays for proteins may not be used in a physiologically relevant ionic strength for the charge detection system due to its relatively large size of tens nanometers (Bukar et al., 2014; Stern et al., 2007b). Therefore, most of the field effect-based immunosensors are operated at low salt concentrations of typically less than 1 mM (Elfstrom et al., 2008; Im et al., 2007; Kim et al., 2010; Lin et al., 2010; Stern et al., 2007a, 2010; Tang et al., 2009; Zheng et al., 2005). A fragmented antibody may be

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http://dx.doi.org/10.1016/j.bios.2015.05.067
0956-5663/

alternatively used as a small capturing element (Elnathan et al., 2012; Kim et al., 2008b), but they generally impose reduced binding affinity and selectivity.

Aptamer is a new class of molecular recognition element composed of a selected short sequence of peptides or nucleic acids by screening through the process called systematic evolution of ligands by exponential enrichment (SELEX) (Darmostuk et al., in press; Stoltenburg et al., 2007). The engineered aptamer is an alternative to antibody with advanced features on chemical and thermal stability, equal quality, and ease of structural design and chemical modification (Famulok and Mayer, 2011; Li et al., 2010; Palchetti and Mascini, 2012; Zhou et al., 2010). Importantly, its small size of a few nanometers comparable to the thickness of electrical double layer allows the target recognition event close to the gate surface without shielding the charges by mobile counterions (Goda and Miyahara, 2012a, 2013; Lee et al., 2008). Recent developments have realized the discovery of many aptamers showing the excellent affinity in terms of the dissociation constant (K_d) at subnanomolar or nanomolar levels for various target species (Jayasena, 1999; Taylor et al., 2014). Nevertheless, the binding ability is generally superior in antibody to aptamer.

In parallel to the continuous challenges in exploring better aptamers, an effort should be made for developing known aptamer-based interface with improved affinity to allow ultrasensitive biosensing towards early cancer diagnosis and single molecule detection. In biological systems, multivalent interactions are involved for producing a high affinity in molecular recognition (Mammen et al., 1998). Learning from nature, we designed biointerfaces to capture protein via cooperative interactions using existing anti-protein aptamers targeting different epitopes. The use of different aptamer sequences for targeting different binding sites in single analyte is feasible since the SELEX process can pick up several candidate sequences at a time (Darmostuk et al., in press; Stoltenburg et al., 2007). In a model study, we demonstrated the concept through the interaction between thrombin and antithrombin aptamers. Thrombin is a serine protease pro-coagulant factor by converting soluble fibrinogen to insoluble fibrin that agglutinates platelets at the site of tissue injury. Thrombin is recognized as a biomarker for several diseases because thrombin is a critical mediator of coagulation, inflammation, and angiogenesis, leading to the development of atherosclerosis, progression of cancer, and growth of tumor (Borissoff et al., 2009; Nierodzik and Karpatkin, 2006). Due to the importance of clinical diagnostics of thrombin, researchers have employed many sensing techniques including electrochemistry (Wang et al., 2009), impedance spectroscopy (Loo et al., 2012), electrochemiluminescence (Jie and Yuan, 2012), colorimetry (Liang et al., 2011), fluorescent spectroscopy (Kong et al., 2013), surface plasmon resonance (Jalit et al., 2013), and so on. Here, we performed a fine control of molecular structure of the aptamers to investigate the factors that may facilitate the multivalent binding such as immobilization format and spacer/linker lengths. Possibility and limitation are discussed in developing a dual aptamer-based nanointerface for improved affinity through bivalent attachments in the FET-based potentiometric biosensing.

2. Materials and method

2.1. Materials

Thrombin from human plasma was obtained from Calbiochem (Merck Millipore, Billerica, MA, USA). Thrombin-binding DNA aptamers with 5′-SH-$(CH_2)_6$-functionalization were purchased from Tukuba Oligo Services (Tsukuba, Ibaraki, Japan). Sulfobetaine-3-undecanethiol (SB) was obtained from Dojindo (Mashiki, Kumamoto, Japan). All the other reagents with extra pure grades were from commercial sources and were used without further purifications. Screen-printed 10-channel gold microelectrode with 500 μm in diameter was formed on a glass-epoxy resin chip and the remaining wiring circuit on the chip was insulated by coating with a SU-8 photoresist.

2.2. Self-assembled monolayer (SAM) formation

The thiol-modified DNA aptamers (2 or 10 μM in final concentration) were immobilized on a 10-channel gold microelectrode array in a reaction buffer (10 mM Tris(hydroxymethyl)aminomethane–hydrochloride (Tris–HCl), 100 mM KCl, 100 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)) for overnight, followed by rinse with water. The remaining gold surface was backfilled by immersing in 1 mM SB aqueous solution containing 1 mM TCEP, followed by rinse with water. The modified electrodes were kept dry in dark at 4 °C until use. The SAM film on the electrode was stable during the measurement for several hours or under the storage in a deoxidized environment for at least one week.

2.3. Electrochemical measurements

Cyclic voltammetry (CV) and chronocoulometry (CC) were performed using an Autolab PGSTAT302 Potentiostat (Metrohm, Herisau, Switzerland). A planar gold thin-film modified with SAM as a working electrode, a platinum wire ($\varphi = 1$ mm) as a counter electrode, a silver/silver chloride wire as a reference electrode in 3.3 M KCl solution with a salt bridge were used. CV scans were performed from −0.1 to −1.3 V at the scan rate of 0.5 V/s in degassed 0.5 M potassium hydroxide solution. CC was conducted in 1 mM Tris–HCl buffer (pH 8.0) with and without 50 μM hexamine ruthenium(III) chloride (RuHex) under the double potential step from 0 V to −0.5 V (for one second) to 0 V.

2.4. Potentiometry

The potentiometric response of the SAM-functionalized gold electrode as a function of varying thrombin concentration was determined using a high-input impedance electrometer (Keythley 6517B, Cleveland, OH, USA) at no DC bias voltage versus the Ag/AgCl (in 3.3 M KCl solution with a salt bridge) reference electrode. Electrolyte composition of measurement buffer (pH 8.2) was 10 mM Tris–HCl, 5 mM KCl, 3 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. For measurements at pH 6.0, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) was used instead of 10 mM Tris–HCl. Aptamers immobilized on the electrode were denatured at 100 °C in the measurement buffers and then were annealed by cooling down to 25 °C, followed by incubation for overnight. The potential of 10-channel working electrodes were stabilized for initial 150 min in the measurement buffer, thereafter the potentials were recorded by increasing thrombin concentrations (0–1300 nM) for each 10 min at 25 °C. Data were represented as mean ± standard deviation (SD) from three sets of the 10-channel microelectrode measurements. The limit of detection (LOD) was based on 3 SD. Sensor stability was evaluated by relative standard deviation (RSD) in percent from a dataset of the 10-channel microelectrode measurements.

2.5. Apparent binding constant

Binding parameters such as the apparent binding constant (K_d) of thrombin onto the SAM-modified surface were determined by fitting the potentiometric signals as a function of thrombin concentration using 1:1 Langmuir adsorption model. Reproducibility