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Effects of redox label location on the performance of an electrochemical aptamer-based tumor necrosis factor-alpha sensor

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ABSTRACT

We report the development of an electrochemical aptamer-based sensor for real time detection of tumor necrosis factor-alpha. The focus of this study is to evaluate the effects of the redox label location on the overall sensor performance, including sensor stability, detection limit, reusability, and selectivity. Three aptamer probes, each labeled with methylene blue (MB) at a specific location, were designed and employed in the fabrication of the sensors. Among the three sensors, the sensor fabricated using an aptamer with the MB label located at the distal end has a detection limit of 100 pM and is regenerable. The sensor fabricated using an aptamer with an internal MB modification has a detection limit of 10 nM and is not regenerable. Both sensors can be employed in complex biological samples such as 50% urine and 50% saliva. However, the sensor fabricated with an aptamer with the MB label located at the proximal end suffers from poor reproducibility and is highly unstable, thus limiting its application as a sensor. On the bases of these results, placing the MB label at the distal end of the aptamer probe appears to be the most advantageous for this sensor design for it does not interfere with monolayer formation and target binding.

1. Introduction

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine known to regulate a host of functions in mammals [1]. It has been recognized to play multiple roles in the body, including the formation, regulation and regeneration of nerves [2], enhancement of immune responses for various degenerative diseases, initiation of gene transcription within cells, and the reduction of tumors [3,4]. Rapid, selective and sensitive detection of TNF-α is of particular importance in disease diagnosis and subsequent therapy monitoring. To date, various TNF-α detection techniques have been reported, including enzyme-linked immunosorbent assays (ELISAs), immunohistochemistry, and radioimmunoassays [5,6]. Although ELISA assays are often considered the gold standard for protein detection, they are not ideal for real-time clinical diagnostics. They are multistep processes and are relatively reagent-intensive. Thus, there are needs to develop new assays for real-time analysis of clinically important proteins such as TNF-α.

Since its inception in the 1990s, aptamers have been widely used in the development of biosensors [7–9]. Aptamers are single-stranded DNA or RNA molecules that can bind to pre-selected targets such as proteins, small molecules, and metal ions with high affinity and specificity [7,8]. Unlike antibodies used in ELISA assays, aptamers are more stable under a wider range of experimental and storage conditions, thus sensors and diagnostic assays that utilize aptamers as biorecognition elements, in general, have a longer shelf life. In addition, aptamer generation is significantly easier and more cost-effective than the production of antibodies. Owing to the merits associated with the use of aptamers in designing sensors, aptamer-based sensors for detection of targets such as thrombin, platelet-derived growth factor, interferon gamma, and vascular endothelial growth factor have since been developed [10–12]. Among the currently available aptamer-based sensors, one specific class of electrochemical aptamer-based (E-AB) sensors has shown to be more promising for real world applications [12–17]. The sensing mechanism of E-AB sensors is based on the change in the conformation and/or the flexibility of the aptamer probe in the presence of the target analyte. This change alters the electron transfer behavior of the attached redox label, resulting in an increase or decrease in the redox current. Despite the number of E-AB sensors available for detection of thrombin, platelet-derived growth factor, and interferon gamma, there are limited reports on E-AB TNF-α sensors [17–19]. Thus, there is a need for sensors capable of real time detection of TNF-α, specifically, in complex biological samples.

Unlike previously reported TNF-α sensors [20,21], the current study focuses on the use of a 25-base DNA aptamer as the biorecognition probe [22]. This probe has not been employed in the design and fabrication of an E-AB TNF-α sensor before. The main goal of this study,

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however, is to determine the effects of the redox label location on the overall sensor performance. While this aspect has been addressed in the fabrication of electrochemical DNA (E-DNA) sensors, it has not been reported for E-AB sensors [23]. According to a previous study on E-DNA sensors, addition of a redox label, methylene blue (MB), in the middle of the DNA probe sequence is advantageous for both stem loop and linear probe sensor designs. The sensing mechanism remains unaffected, but the signal attenuation is larger [23]. Peptides used in the fabrication of electrochemical peptide-based (E-PB) sensors have also been modified with a redox label at different locations [24–27]. These sensors were reported to be functional independent of the location of the redox label. The effects of redox label location on the performance of E-AB sensors, specifically, an E-AB TNF-α sensor, are not known. Thus, in this study we aim at addressing this important issue by providing a detailed comparison between three different E-AB TNF-α sensors, each fabricated using an aptamer that is labeled with MB at a specific location. The results from this study could provide insights into the design of not only E-AB TNF-α sensors but also other E-AB protein sensors.

2. Experimental

2.1. Materials and reagents

Glutathione hydrochloride (GHCl), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), 4-mercapto-1-butanol (C4-OH), 6-mercapto-1-hexanol (C6-OH), sulfuric acid (H_2SO_4), sodium chloride (NaCl), sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic, 10% sodium dodecyl sulfate solution (SDS), creatinine, and TRIS hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. TNF-α was purchased from Shenandoah Biotechnology Inc. Protein Pros (Warwick, PA). Synthetic human stimulated parotid saliva was purchased from US Biocontract (San Diego, CA). Synthetic urine solution was purchased from Ricca Chemical Company (Arlington, TX). Creatinine (11 mM) was added to the synthetic urine solution prior to being used in the experiments. All other reagents were of analytical grade and used as received. The three thiolated and MB-modified aptamers were purchased from Biosearch Technologies (Petaluma, CA). The three probe sequences are as follows [22]:

A1:
5′HS-$(CH_2)_6$-TCCTCATATAGAGTGCGGGGCGTGT(**MB**) 3′
A2:
5′HS-$(CH_2)_6$-TCCTCATAT(**MB**)AGAGTGCGGGGCGTGT 3′
A3:
5′HS-$(CH_2)_6$-T(**MB**) CCTCATATAGAGTGCGGGGCGTGT 3′

The structures of the aptamers are shown in Fig. S1. The secondary structures of the aptamer were determined using Mfold, a web server for nucleic acid folding and hybridization prediction (Fig. 1D) [28]. The sensors were interrogated in a pH 7.4 buffer with 10 mM sodium phosphate and 100 mM NaCl (PBS), 1:1 urine:PBS, and 1:1 saliva:PBS. All solutions were made with deionized water (DI H_2O) purified through a Millipore Synergy system (18.2 MΩ cm, Millipore, Billerica, MA).

2.2. Sensor preparation and characterization

Prior to sensor fabrication, the gold working electrodes were mechanically polished using 0.1 μm diamond suspension purchased from Buehler, Inc. (Lake Bluff, IL). The electrodes were then sonicated using an ultrasonic bath for ~ 5 min to remove the adsorbed or bound polishing materials. The electrodes were then electrochemically cleaned by repeated scanning from − 0.2 V to 1.3 V at 4 V s^{-1} in 0.5 M H_2SO_4 until no change in the gold oxide formation and reduction currents was observed. The electrodes were then placed in 50 mM H_2SO_4, and cyclic voltammograms (CVs) were recorded at a scan rate of 0.1 V s^{-1}. The charge under the cathodic peak at ~ 0.85 V in the CV was used to determine the actual area of the electrode. The ρ factor (actual area/geometric area) of the electrodes used in this study was between 1.0 and 1.4. The electrodes were rinsed with DI H_2O and dried with N_2 gas prior to sensor fabrication.

Fabrication of the **A1** and **A2** sensors involved two steps. The aptamer stock solution (1 μL at 0.2 mM) was first mixed with TCEP (1 μL at 10 mM) for 1 h to reduce the disulfide bond. This solution was then diluted with PBS to achieve a concentration of 1 μM. The cleaned electrodes were placed in this diluted aptamer solution for 1 h in a 4 °C refrigerator. They were then rinsed with PBS and placed in a 2-mM C4-OH solution for ~ 5 h in a 4 °C refrigerator. Fabrication of the **A3** sensor also required a two-step approach, but the concentration of the aptamer probe was 0.5 μM and the electrodes were passivated in 2 mM C4-OH for 16–20 h. After these two steps, the sensors were rinsed with PBS and placed in an electrochemical cell.

All electrochemical measurements were performed in a conventional three-electrode system using a CHI 1040 A Electrochemical Workstation (CH Instruments, Austin, TX) [30,31]. Polycrystalline gold disk electrodes (CH Instruments, Austin, TX) with a diameter of 2 mm (geometric area of 0.0314 cm^2) were used as working electrodes. A Ag/AgCl (3 M KCl) electrode and a platinum wire electrode (both from CH Instruments, Austin, TX) were used as the reference electrode and counter electrode, respectively. The sensors were characterized using both alternating current voltammetry (ACV) and cyclic voltammetry (CV) [30].

The probe density was determined using the charge under the MB reduction peak in CV scans collected at slow scan rates (20, 50 and 100 mV s^{-1}) in PBS. The following equation (Eq. (1)) was used to calculate the probe density.

$$\Gamma = Q/nFA \quad (1)$$

where Γ is the density of the aptamer probe, Q is the charge associated with the MB reduction peak in the CV scans, n is the number of electrons transferred in the electrochemical reaction ($n = 2$ for MB), F is the Faraday's constant, and A is the real electrode area.

A series of CV scans were recorded both before and after target interrogation to determine k_s for MB. The peak potential separation ($\Delta E_p = E_{p,a} - E_{p,c}$) increases with scan rate (ν). When ΔE_p is > 200/n mV, plotting ΔE_p versus log ν yields a straight line, which is in accordance with the Laviron equation (Eq. (2)) [29]:

$$\text{Log } k_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log(RT/nF\nu) - \alpha(1-\alpha)nF\Delta E_p/2.3RT \quad (2)$$

where k_s is the electron transfer rate constant (s^{-1}), α is the electron transfer coefficient, ν is the CV scan rate (V/s), and ΔE_p is the peak-to-peak separation (V). The α value can be determined from the slope of the straight line, and k_s can be calculated using the intercept [30].

2.3. Target interrogation and regeneration

Prior to target interrogation, the sensors were allowed to equilibrate in PBS until a stable MB peak current was obtained in ACV. Each sensor was characterized through electrochemical interrogation with 100 nM TNF-α. ACVs were collected after target addition until the change in the MB current was less than 0.5%. For the **A1** sensor, sensor regeneration was achieved by incubating the sensor electrode in 10% SDS for 4 min. The regenerated sensor was rinsed with DI H_2O prior to being placed in a new aliquot of buffer. Despite multiples attempts and the use of other regeneration reagents, neither **A2** nor **A3** sensors were successfully regenerated. Both **A1** and **A2** sensors were also interrogated in 1:1 saliva: 2xPBS (20 mM PBS with 200 mM NaCl), and 1:1 urine:PBS. The final concentration of creatinine was 5.5 mM in the diluted urine sample. ACVs were collected until the change in the MB current was less than 0.5%. Calibration curves were obtained in PBS in ACV. For the **A1** sensor, the concentrations of TNF-α used were 0.1, 0.5, 1, 10, 50,