Electrochimica Acta 54 (2009) 6788–6791

Contents lists available at ScienceDirect

Electrochimica Acta

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

Aptamer-based electrochemical detection of protein using enzymatic silver deposition

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ARTICLE INFO

Article history:
Received 21 May 2009
Received in revised form 22 June 2009
Accepted 27 June 2009
Available online 5 July 2009

Keywords:
Aptamer
Protein
Linear sweep voltammetry
Thrombin
Silver deposition

ABSTRACT

In this work an electrochemical protein detection based on enzymatic silver deposition has been proposed and applied to the detection of thrombin. The target protein, thrombin, was first captured by thrombin binding thiolated aptamer self-assembled monolayers (SAMs) on the gold electrode surface, and then sandwiched with another biotinylated thrombin binding aptamer for the association of alkaline phosphatase (Av-ALP). The attached Av-ALP enzymatically converts the nonelectroactive substrate *p*-aminophenyl phosphate (*p*-APP) to *p*-aminophenol (*p*-AP) which can reduce silver ions in solution leading to deposition of the metal onto the electrode surface. Finally, linear sweep voltammetry (LSV) is used to detect the amount of deposited silver. The peak current during the anodic scan was found to reflect the amount of the target protein captured into the sandwich configuration. The proposed approach has been successfully implemented for the detection of thrombin in the range of 0.1 nM to 1 μM. Therefore, the current work has demonstrated enzymatic silver deposition for detection at aptamer-modified electrode.

1. Introduction

The detection and quantification of proteins play essential roles in fundamental research as well as in clinical practice. To date, antibody-based immunological assays are the most commonly used methods for protein detection, but recent reports have demonstrated the utility of protein binding nucleic acids, aptamers, as new alternative molecular recognition elements in the design and development of biosensors for protein detection [1–3].

Aptamers are single-stranded DNA or RNA molecules, selected from large pools of random-sequence oligonucleotides due to their ability to bind certain non-nucleic acid targets with high affinity and specificity. The methodology for the selection of these aptamers, coined as SELEX (Systematic Evolution of Ligands by EXponential enrichment), was independently reported by three laboratories in 1990 [4–6]. It consists of an exponential enrichment of a nucleic acid library via an *in vitro* iterative process of adsorption, recovery and re-amplification. The first feasibility of SELEX-derived high affinity oligonucleotide ligand as a biorecognition element was reported in 1996, with an optical biosensor based on fluoresceinated DNA [7]. Then, a significant number of aptamers have been identified and described to bind an extensive variety of targets such as metal ions [8,9], organic molecules [10], peptides [11], proteins [12–15] and even whole cells [16]. Moreover, due to their distinctive properties, such as high association constant with target, easy production, easy labeling with signal moieties, cost-effectiveness and stability during long-time storage, aptamers have attracted the attention of many research groups for fundamental studies and as well as for practical applications [1–3].

Thus, to this end, recent years have witnessed substantial progress on the understanding of nucleic acids in terms of their conformation and ligand binding properties leading to the use of these two properties for the development of new generation of aptamer-based sensors, aptasensors, based on different signal transduction methods such as electrochemical, mass or optical detection [1–3,17–21]. The electrochemical transduction-based aptasensors present considerable advantages such as portability, low-cost and simplicity-to-operate, robust and easily miniaturized over that of piezoelectric, optical, or thermal-based aptasensors.

The first electrochemical aptasensor was described in 2004 [22], with an amperometric sandwich-based on glucose dehydrogenase-labeled signaling aptamer. Since these initial report, several electrochemical aptasensors have been reported mostly based on: (i) control of the electron transfer between the electroactive moieties, such as ferrocene and methylene blue, covalently linked to the terminal of the aptamer and the electrode, with the conformational change of the aptamers induced by specific target binding [23–25], (ii) evoking a change in the current signal or

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doi:10.1016/j.electacta.2009.06.082

charge transfer resistance induced up on specific target binding to the ss-DNA aptamer assembled on the electrode [26–28] or with unwinding of the ds-DNA, i.e., aptamer–complementary DNA, assembled onto the electrode and subsequent dissociation of either the aptamer or complementary DNA oligonucleotide from the electrode surface up on binding target [29,30], and (iii) a sandwich assay using a pair of aptamer, serving as capture and detection probes for capturing of the target protein [22,31–33], and subsequent detection with enzymes labels including horseradish peroxidase, streptavidine–alkaline phosphatase and glucose dehydrogenase or nanoparticles such as gold, silver, platinum, and quantum dots [1–3].

Even though the use of a pair of aptamer have been reported with different detection strategies for ultrasensitive optical and electrochemical aptasensors [1–3], there is no report demonstrating protein detection using aptamer as a sensing layer and enzymatic deposition of silver as a detection strategy except the recent report by Yu and co-workers [34,35] which relies on uses antibody to capture the protein target. Our group originally reported a novel electrochemical detection of DNA hybridization based on the enzymatic deposition of silver, a process termed as biometallization [36]. The goal of the present study is to elucidate the feasibility of using enzymatic deposition of silver as a means for electrochemical detection of protein using a pair of aptamers, one self-assembled on a gold electrode as a sensing layer, the second serving as a detection probe with enzyme label, followed by enzymatic deposition of silver ions and linear sweep voltammetric read out signal. These approaches will circumvent the difficulties such as antibody labeling, high cost and time consuming encountered in the classical antibody-based immunoassay format involving antibody immobilization to capture target protein and a secondary antibody labeled with an enzyme for detection.

2. Experimental

2.1. Chemicals and reagents

Silver sulfate (99%) and mercaptoethanol (MCE, 98%) were purchased from Aldrich (Seoul, Korea). Thrombin from human plasma, avidin from egg white, albumin bovine serum (BSA) and lysozyme from chicken were obtained from Sigma (Seoul, Korea). Neutravidin-conjugated alkaline phosphatase (Av-ALP) was obtained from Pierce (Rockford, IL). *p*-Aminophenyl phosphate (*p*-APP) was purchased from Biosynth (Switzerland). All buffer salts and other inorganic chemicals were obtained from Sigma or Aldrich unless otherwise stated. All chemicals were used as received. Ultrapure water (>18 MΩ from a Modulab water system, U.S. Filter Corp.) was used throughout this work. Solutions of *p*-APP and Ag_2SO_4 were prepared freshly and protected from light.

2.2. Aptamers and proteins

The aptamers used in this study were synthesized and purified using an HPLC apparatus supplied by Geno-tech (Daejeon, Korea). Two different thrombin binding DNA aptamers, a primary aptamer 5′-GGT TGG TGT GGT TGG-3′ (aptamer-I) as a capture probe and a secondary aptamer 5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′ (aptamer-II) as a detection probe were used. It has already reported that these aptamers recognize different parts of thrombin [22]. The primary aptamer was 5′-thiolated with C6 to achieve immobilization on the gold substrate and a 6-T (thymine) spacer was inserted between the C6 and the oligomer. The secondary aptamer was 3′-labeled with biotin group and a 5-T (thymine) spacer was inserted between the oligomer and the biotin. Thrombin for the analysis of specific interaction, and bovine serum albumin (BSA), avidin and lysozyme for the analysis nonspecific interactions were used as model proteins.

2.3. Electrode preparation

Gold electrodes were prepared by electron-beam evaporation of 40 nm of Ti followed by 150 nm of Au on Si (100) wafers. The electrode was cleaned in piranha solution (3:7 (v/v) 30% H_2O_2/70% H_2SO_4), followed by copious rinsing with distilled water (*Warring*: *piranha reacts violently with organics*). It was then dried with nitrogen gas.

2.4. Electrochemical measurements

Electrochemical measurements were performed using CHI900B electrochemical analyzer (CH Instruments, Austin, TX) interfaced with a personal computer. A standard three-electrode cell was used with aptamer-modified gold plate as a working electrode, $Hg|HgSO_4|K_2SO_4$ (sat) electrode (MSE) as a reference electrode and a Pt wire counter electrode. Linear sweep voltammetry (LSV) was performed in 0.1 M H_2SO_4 solution deoxygenated with Ar prior to use.

2.5. Preparation of oligonucleotide aptamer and mixed monolayers on gold surface

Immobilization of the oligonucleotides was based on a previously reported protocol [26] by incubating a clean gold substrate in 1 μM thiolated oligonucleotide aptamer-I solution in a potassium phosphate buffer (0.5 M, pH 7.0) for overnight followed by rinsing with rinsing buffer (0.5 M phosphate buffer, pH 7.6), distilled water and drying under a stream of nitrogen. To reduce nonspecific adsorption, the surface of the gold substrate was blocked by 1 h treatment with 1 mM 2-mercaptoethanol (MCE). Then it was rinsed with rinsing buffer, distilled water and dried under a stream of nitrogen.

2.6. Sandwich aptamer–protein assay

The aptamer-I-modified gold substrates were incubated for 30 min with the desired amount of target protein in a binding buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$, pH 7.4) followed by rinsing with protein rinsing buffer (50 mM phosphate buffer, 0.1% Tween 20 and 1% BSA; pH 7.6), distilled water and drying under stream of nitrogen. Then, the gold substrates were incubated with the biotinylated detection probe aptamer-II (1 μM) for 1 h, followed by rinsing with rinsing buffer, distilled water and drying under a stream of nitrogen.

2.7. Association of Av-ALP

The resulting aptamer–protein–aptamer sandwich assembly was incubated in a binding buffer (50 mM Tris–HCl and 1 mM $MgCl_2$, pH 8) containing 10 μL/mL Av-ALP for 5 min. Then, the electrode was immersed for 20 min in protein rinsing buffer to prevent the nonspecific adsorption of protein, followed by rinsing with distilled water and drying under a stream of nitrogen.

2.8. Enzymatic deposition of silver

The electrode was incubated with freshly prepared 50 mM glycine buffer solution (pH 9.0) containing 0.5 mM Ag_2SO_4 and 1 mM *p*-APP for 30 min, protected from light. Then, it was rinsed with phosphate buffer (pH 7.6) followed by distilled water and dried under a stream of nitrogen.

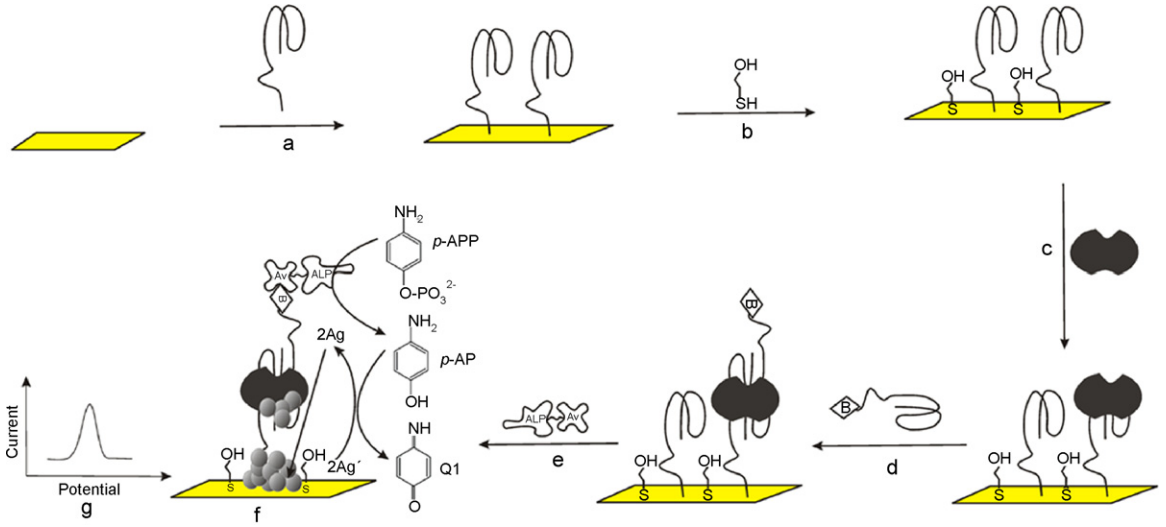

Fig. 1. Schematic outline of electrochemical protein detection based on enzymatic silver deposition. (a) Immobilization of thiolated protein binding aptamer-I on the Au electrode; (b) mixed monolayer of mercaptoethanol (MCE) on the aptamer-I immobilized electrode; (c) capture of target protein; (d) binding with the detection probe biotinylated aptamer-II; (e) association with Av-ALP; (f) enzymatic silver deposition; and (g) dissolution of silver during anodic stripping voltammetry.

2.9. Electrochemical detection

Linear sweep voltammetric (LSV) scan were performed in 0.1 M H_2SO_4 using a standard three-electrode cell system with the aptamer-modified gold plate as a working electrode, a $Hg|HgSO_4|K_2SO_4$ (sat) (MSE) reference electrode and a Pt wire counter electrode. The silver stripping peak current read out was related to the concentration of target protein.

3. Results and discussion

Fig. 1 depicts schematically the protocol used for the electrochemical detection of protein based on the enzymatic deposition of silver. Briefly, mixed monolayer surfaces of aptamer-I and 2-mercaptoethanol (MCE) were prepared by exposing a cleaned gold substrate to a solution of thiolated aptamer-I, followed by immersion into mercaptoethanol (MCE) solution. The immobilized aptamer-I was used to capture the target protein and afterward a sandwich-type, aptamer-I/protein/aptamer-II, configurations were obtained by reacting with biotinylated aptamer-II. The sandwich configuration was completed by the binding of avidin conjugated alkaline phosphatase (Av-ALP) to the exposed biotin group on the aptamer-II. The Av-ALP allowed a non-reductive substrate of Av-ALP, *p*-aminophenyl phosphate (*p*-APP) to be converted into *p*-aminophenol (*p*-AP), a reducing agent that reduces silver ions to form silver metal on the electrode surface and backbone of the DNA [34–37]. The reduction can be explained by the following equations:

$$p\text{-AAP} \xrightarrow{\text{Enzyme}} p\text{-AP}$$

$$2Ag^{+} + p\text{-AP} \rightarrow 2Ag + QI$$

where QI (quinoimide) is the oxidation product of *p*-AP. Therefore, only the enzymatic reaction can start the reduction of silver ions in solution. Thus, the peak current during the anodic linear sweep voltammetric scan provides the amount of target proteins.

Fig. 2 shows the linear sweep voltammetric (LSV) response for the electrochemical detection of thrombin based on the enzymatic deposition of silver. A well-defined peak corresponding to the silver stripping is observed at ~0.05 V versus MSE [36]. The response was recorded for different concentration of thrombin over the range of 0.1 nM to 1 μM. It is clear from Fig. 2I that the sensor response increases substantially when the thrombin concentration is increased. The plot of the peak current versus target protein concentrations in log scales is linear over the considered range (Fig. 2II). It is noteworthy that the current detection limit is comparable

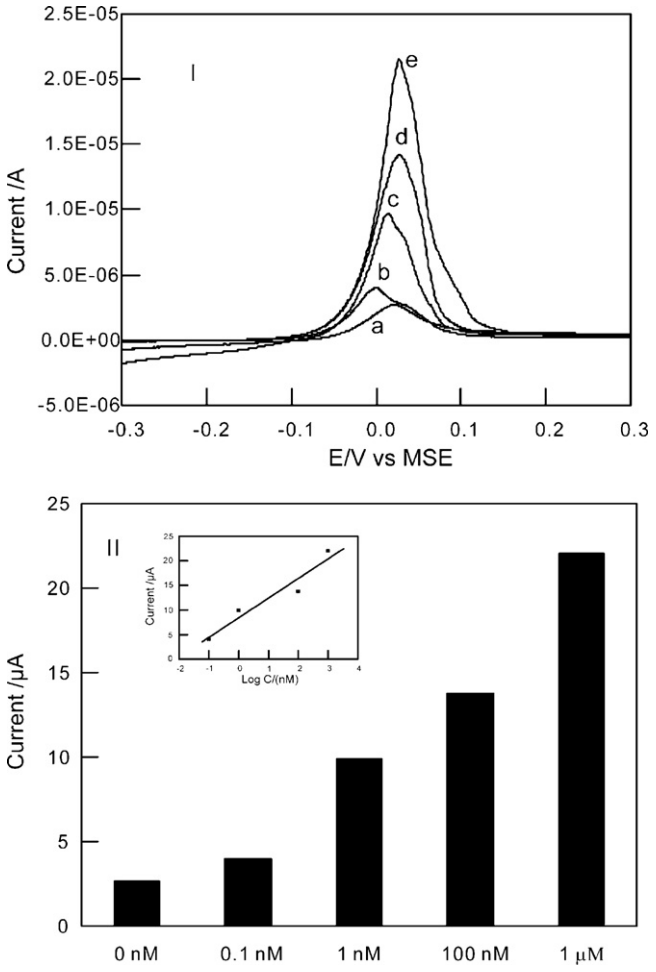

Fig. 2. (I) LSV at aptamer-I/thrombin/aptamer-II with: (a) 0 nM, (b) 0.1 nM, (c) 1 nM, (d) 100 nM, and (e) 1 μM thrombin. (II) Plot of the stripping peak current with different concentrations of the target protein from the LSVs in (I).

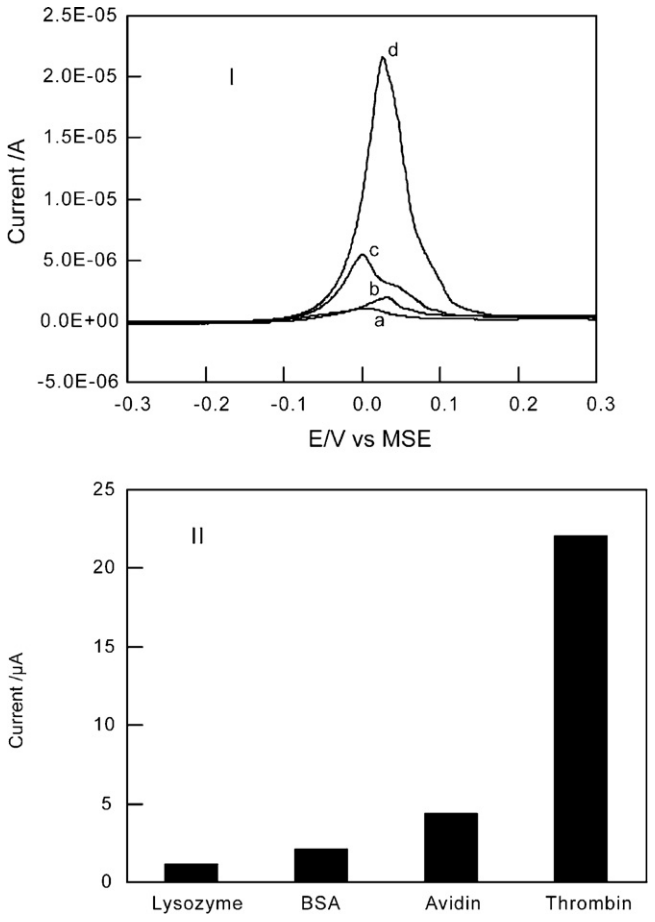

Fig. 3. (I) LSV at aptamer-I/protein/aptamer-II with 1 μM of (a) lysozyme, (b) BSA, (c) avidin, and (d) thrombin. (II) Plot of the stripping peak current with different protein (1 μM) from LSVs in (I).

with previously reported electrochemical aptasensors for thrombin [1–3], and can be further lowered by optimizing the enzymatic reaction conditions [37–40].

The selectivity of the electrochemical protein detection based on the enzymatic silver deposition was also examined using other proteins (BSA, avidin and lysozyme) in control experiments. It can be seen from Fig. 3 that the signal obtained for thrombin is significantly larger than the signals for the same concentration of BSA, avidin and lysozyme. This observations show that in the presence of thrombin, the secondary aptamer could be more successfully captured on the electrode surface and will allow the association of Av-ALP for the subsequently execution of the enzymatic reaction. The small signal observed in the presence of the nontarget proteins can be attributed to unspecific binding of protein [31,35]. Thus, the proposed protein detection based on aptamer-modified electrode and enzymatic silver deposition detection strategy has good recognition selectivity.

4. Conclusion

In summary, in this report we elucidate the feasibility of using enzymatic deposition of silver (biometallization) for the electrochemical detection of protein at gold electrode modified with thiolated aptamer self-assembled monolayers (SAMs) to capture a target protein and a detection biotinylated aptamer probe for the realization of a sandwich, aptamer–protein–aptamer, configuration and subsequent association of Av-ALP for the execution of the enzymatic reaction. The enzymatic reaction and the enzymatic deposition of the silver are initiated where there is attached biotinylated aptamer probe. The deposited silver can de detected through the peak current when an anodic stripping scan is carried out. The peak current indicates the capture of the target protein responsible for binding of the biotinylated aptamer in the sandwich configuration and can be correlated to the concentration of the protein. Thus, considering the increasing availability of aptamers for a number proteins and the increasing interest in the aptasensors [1–3], this report highlights a new direction for the electrochemical detection of protein by making use of the advantage of specific binding of protein by aptamer and the enzymatic silver deposition.

Acknowledgements

This work was supported by the Nano/Bio Science & Technology Program (2005-01333) of the Ministry of Education, Science and Technology and also supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-314-C00234).

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