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Electrochemical assay of proteolytically active prostate specific antigen based on anodic stripping voltammetry of silver enhanced gold nanoparticle labels

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ABSTRACT

This work demonstrated the determination of proteolytically active prostate specific antigen (paPSA), a potential biomarker for prostate cancer diagnosis, in human serum using a sensitive and low-cost electrochemical sensor. A specifically designed peptide probe was immobilized on the surface of a 96-well plate. The probe could be recognized by paPSA causing cleavage of the peptide, resulting in a decrease in the thiols group remaining on the probe. Gold nanoparticles (AuNPs) were attached to the peptide thiol groups by self-assembly. Hence the amount of AuNPs relates to the length of peptide probe. After cleavage and binding of AuNPs, an amplification step was performed using a silver enhancer solution. The quantity of deposited silver was then measured by differential pulse anodic stripping voltammetry (DPASV) using a disposable screen-printed carbon electrode (SPCE). The signal for paPSA detection was the linear range from 0.1 to 100 ng mL^{-1}, with a detection limit of 27 pg mL^{-1}. We also showed that the assay was reliable and has potential for clinical applications.

1. Introduction

Prostatic carcinoma (PCa) ranks as the second most common cancer and the most common malignant tumor in men [1,2]. Total prostate specific antigen (PSA), a serine protease, in blood has been screened for PCa diagnosis. Serum containing 4–10 ng mL^{-1} PSA (diagnostic grey zone) is generally regarded as the cutoff for whom a prostate biopsy should be performed, although the specificity of the test is poor when PSA values are below 10 ng mL^{-1} [3,4]. PSA levels can also be elevated with benign conditions such as benign prostatic hyperplasia, prostatitis, ejaculation and minor trauma to the prostate, leading to high false-positive rate in this regard [3,5,6]. The situation of "overdiagnosis" or "pseudo-disease" therefore occurs frequently in men who have asymptomatic PCa. Recently, the U.S. Preventive Services Task Force (USPSTF) discouraged PSA-based screening for PCa [7].

It is known that two general forms: free-floating PSA (fPSA) and PSA complexed to protein (cPSA) exist in blood serum. PCa tissue produces more PSA and it may leak into the circulation and turn into complexed forms. Therefore, a number of researches suggest that determining the ratio between fPSA and total PSA (tPSA) might improve the specificity more than tPSA alone [8,9]. As such, many attempts have been made to apply fPSA/tPSA in advanced PCa diagnosis [10–12]. According to this, multiplex assays based on dual sensor [13], dual label [14], and shifted capacitance [15] were developed to facilitate a measurement of f/tPSA. However, the clinical utility of tPSA and f/tPSA measurement is still controversial with issues such as cutoff value for prostate biopsy and specificity over benign conditions [16–19]. Other biomarkers that can distinguish between indolent and high-grade disease have also been sought such as, fPSA isoforms, PSA density and PSA velocity [20,21]. Among these markers, proteolytically active forms of PSA (paPSA) as an isoform of fPSA, is the most attractive due to its relevant enzymatic activity to PCa. It is well documented that paPSA, a serine protease, could be involved in cancer cell invasion [22] and metastasis [23]. The paPSA confers an enhanced growth rate to a human PCa cell [24], indicating a causal role in PCa aggressiveness [25].

Enzymatically cleavable peptide sequences have been developed for the detection of paPSA with a high degree of specificity and good stability, because it has not been possible to determine it directly by immunoassay [26–28]. There are several peptide-based methods for the measurement of paPSA, including immunopeptidometric assay [29], surface enhanced Raman scattering [30], fluorescence [31,32], electrochemiluminescence [33], and colorimetric assay [34]. Electrochemical methods have attracted

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considerable attention as it enables direct electronic readout and miniaturization with cost effective and potentially high sensitivity [35]. A few reports have presently addressed electrochemical labels for paPSA detection based on peptide-cleavage. Zhao et al. used a ferrocene-peptide probe for direct electrochemical measurement of peptide cleaves events into a signal related to the activity of paPSA [36], the given technique was simple and easy but produced poor sensitivity. Electrochemical labels for high sensitivity down to pg mL^{-1} have been reported such as using the host-guest interaction between β-cyclodextrin and ferrocene [37], dithiobis(succinimidylpropionate) (DPS)@Au@SiO_2 [38].

Here, we report an alternative, relatively simple and low-cost, electrochemical peptide cleavage method working in microplate. The electrochemical signals were derived from the stripping analysis of silver ion, dissolved from silver deposited on AuNPs label. In the presence of paPSA, the peptide-modified microplate was cleaved and release short peptide tagged thiol group out. Hence the amount of AuNPs binding to the peptide probe in microplate reduced according to provide low signal. With user-friendly of 96-well plate, it can easily handle and measure several samples in the same time. Moreover, silver enhancement process in microwell can reduce the non-specific enhancement directly at silver reference electrode.

2. Materials and methods

2.1. Apparatus

Transmission electron microscopy (TEM) was carried out with a JEOL model JM-2100. UV–visible spectra were recorded using a Beckman model DU-7000 spectrophotometer. Electrochemical experiments were performed using an Autolab PGSTAT 12 computer-controlled potentiostat (Eco Chemie) with GPES software. The screen-printed carbon electrodes (SPCEs; product no. BI1201T3), containing a carbon working electrode and a Ag/AgCl combined reference-counter electrode were obtained from Quasense Co., Ltd. (Bangkok, Thailand).

2.2. Reagents and buffers

Serine proteases (paPSA, chymotrypsin and thrombin), and tri-sodium citrate were from Merck KGaA (Darmstadt, Germany). The peptide substrate, (3-mercaptopropionic acid-HSSKLQ-[K (biotin)]) was purchased from Proteogenix SAS (Schiltigheim, France). Bovine serum albumin (BSA) was purchased from Biobasic Inc. Avidin from egg white, pooled human serum (from human male AB plasma, sterile-filtered), silver enhancer solution, tris(2-chloroethyl) phosphate (TCEP), horse radish peroxidase (HRP) and chloroauric acid ($HAuCl_4 \cdot 4H_2O$) were purchased from Sigma-Aldrich Chemical Inc. (Germany). All reagents were of analytical grade and used as received. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore) was used in all assays.

Fifty millimolar carbonate/bicarbonate buffer (pH 9.6) was used as coating buffer. 10 mmol L^{-1} phosphate buffer (PB), pH 7.4, was used as working buffer. PB containing 0.05 vol% Tween-20 (PBT) and PBT containing 10 mmol L^{-1} TCEP (PBT-TCEP) were used as washing buffer. 5 mmol L^{-1} Tris solution was used for diluting Au nanolabels. Nitrate (0.5 M $NaNO_3$) and nitrate-surfactant (0.5 M $NaNO_3$ including 0.2 wt% and 0.01 wt% SDS) solution were used for washing unbound Au nanolabels. 0.1 mol L^{-1} sodium acetate-acetic, pH 5.5, was used as analysis buffer. A 1:1 (v/v) mixture solution of silver enhancer solution A and B was freshly prepared for silver deposition.

2.3. Preparation of paPSA substrate array plate

A 96-well plate (Nunc MaxiSorp®) was firstly coated with 50 μL of 2.5 μg mL^{-1} avidin in coating buffer overnight at 4 °C. After that, unbound avidin was washed 3 times with 200 μL PBT washing buffer. Unoccupied space was blocked with 300 μL of PB containing 2% BSA and incubated for 30 min at room temperature, followed by washing 3 times with 200 μL of PBT and 2 times with 200 μL of PB. Then, 50 μL of 0.4 μmol L^{-1} paPSA substrate peptide in PB was added and incubated for 30 min at room temperature, followed by washing 3 times with PBT. The peptide substrate array plates were stored at 4 °C in a dry environment prior to use.

2.4. Sensing of paPSA

Each well of paPSA substrate array plates was allowed to react with 50 μL of desired sample (e.g. paPSAs in working buffer or serum) for 1 h at 37 °C, followed by washing 3 times with PBT and 5 times with PBT-TCEP buffer to prevent disulfide bonding (S—S) between peptide substrate. Then, 3 nmol L^{-1} of AuNPs (14 nm in diameters, Fig. 1S), prepared by sodium citrate reduction method [39], were preconcentrated by centrifugation and diluted as desired by Tris buffer solution and 50 μL was delivered into the well for 15 min at 25 °C. After, the excess AuNPs label was removed by washing 3 times with nitrate-surfactant solution and a second time with nitrate solution, to remove Cl^- ions and thus prevent silver chloride (AgCl) formation during the Ag enhancement process [40]. Deposition of silver was carried out by adding 50 μL of silver enhancer solutions (mixed solution A and B at 1:1 ratio) in each well for 4 min under dark conditions, followed by washing with deionized water 3 times before current signals were measured by the electrochemical method.

2.5. Electrochemical measurement

SPCE was immersed into the well after the silver label was dissolved in 50 μL of 50 wt% nitric acid for 2 min and then mixed with 200 μL of analysis buffer. DPASV measurements were performed under a stirred-mixed condition, 300 s electrodeposition time (t_{dep}) at −0.4 V electrodeposition potential (E_{dep}), and then the potential was scanned from −0.3 to 0.7 V at 50 mV s^{-1} scan rate, modulation amplitude 0.05 V, and step potential 0.0063 V.

2.6. Preparation of serum samples

Human serum was applied for paPSA detection in an application in real samples, following the literature [41]. Briefly, different concentrations of paPSA were spiked into the serum. Samples were centrifuged at 13,000 rpm for 15 min to separate lipids and the serum, subsequently removed from the lipid layer. Thereafter, as-prepared serum samples were diluted 10 fold by working buffer. Finally, the paPSA detection was suddenly carried out upon the serum sample preparation by the proposed method. The detected concentration and the relative standard deviation was calculated.

3. Results and discussion

The specific peptide sequence to paPSA was designed as MPA-HSSKLQ-[K(biotin)]. The N-terminal was modified with MPA for a functional thiol (SH) group, which it can bind to AuNPs. HSSKLQ is a paPSA specific recognition sequence [42]. The other end C-terminal was tagged with biotin for immobilization of peptide on the surface of avidin coated 96 well plate via avidin-biotin interaction, using lysine (K) as the linker which not affect the recognition of paPSA activity [33,36]. The proteolytic activity of paPSA detection using peptide modified microplate was performed as shown in Scheme 1 by followed 5 steps: (1) cleaving peptide step by paPSA, (2) binding to gold nanoparticle to the remaining of peptide-SH, (3) silver enhancement, (4) Ag dissolution by 50% HNO_3, (5) quantification of the Ag^+ ions at the bare screen-printed electrode by DPASV ($E_{dep} = -0.4$ V and $t_{dep} = 300$ s). The passive signal will be received which relate to the increasing of paPSA concentration.

To obtain a great analytical performance, parameters affecting sensing performance such as pH of analysis buffer, concentration of AuNPs