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Grafting of a peptide probe for Prostate-Specific Antigen detection using diazonium electroreduction and click chemistry

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ABSTRACT

The main objective of this work was to validate a label-free electrochemical method of protein detection using peptides as capture probes. As a proof-of-concept, we used a 7 amino acids sequence (HSSKLQL) specific for *Prostate Specific Antigen*. We investigated various electrografting conditions of two anilines (2-[(4-aminophenyl)sulfanyl]-8-hydroxy-1,4-naphthoquinone and 4-azidoaniline) further converted *in situ* into their corresponding diazonium salts on glassy carbon electrodes. It was demonstrated that the best method to obtain a mixed layer is the simultaneous electroreduction of the two diazonium salts. 4-azidoaniline was used to covalently immobilize the ethynyl-functionalized peptide probe by click coupling, and the hydroxynaphthoquinone derivative plays the role of electrochemical transducer of the peptide-protein recognition. The proteolytic activity of PSA towards a small peptide substrate carrying streptavidin at its distal end was also investigated to design an original sensing architecture leading to a reagentless, *label free, and "signal-on"* PSA sensor. Without optimization, the limit of quantification can be estimated in the nM to pM range.

1. Introduction

Prostate Specific Antigen (PSA, also named kallikrein-related peptidase-3, KLK3) is well-known as a biomarker of prostate cancer, one of the most common male cancer. Despite its clinical importance, the functional role of PSA in prostate cancer is not well established. It is admitted that a PSA concentration higher than 4.0 ng mL^{-1} is a warning threshold which should trigger a comprehensive medical analysis.

PSA (a 28 kDa glycoprotein) presents enzymatic properties. It is a serine protease with chymotrypsin-like enzymatic activity. Its isoelectric point is 6.9, i.e. slightly negatively charged at physiological pH. Detecting PSA has been routinely performed since the late 80s using immuno-enzymatic techniques. These immunologic techniques based on antibody/antigen interaction constitute the most widely studied form of affinity biosensor. They are now mature, with a large number of publications available (Sarkar et al., 2002, 2008; Okuno et al., 2007; Zhang et al., 2007; Fragoso et al., 2008; Escamilla-Gomez et al. 2009; Gao and Cranston, 2010; Kim et al., 2010; Dey et al., 2012; Li, Dai et al., 2013; Li et al., 2014) and reported limits of detection began from 9 ng mL^{-1} down to 1 pg mL^{-1}, one publication reporting a LOD of 1 fg mL^{-1} (Zhang et al., 2010).

Non-immunologic approaches were poorly investigated yet. Among them, nucleic aptamers were used by Liu et al., 2012, Xia et al., 2013, Souada et al., 2015 and Jolly et al., 2016. Another approach consists in using a stable complex of RNA and peptide in so-called ribonucleopeptides (Hasegawa et al., 2008). However, because PSA is a peptidase (i.e. able to cleave some peptide bonds in proteins), PSA may also be detected throughout its cleaving activity, which presents the advantage to only detect and quantify the truly active PSA. Ferrieu-Weisbuch et al. (2006) first published a molecular biology article reporting several peptide sequences cleaved specifically by PSA. Reported sensors exploiting this strategy were mostly based on fluorescence (Choi et al., 2013; Lee at al., 2013; Gooch et al., 2014), and very few are based on an electrochemical principle. Zhao et al. (2010) presented an electrochemical method to detect PSA using such specific peptide sequences. In particular, they used an 8 amino acids peptide, CHSSKLQK; transduction was performed through an electroactive ferrocenyl moiety coupled on the peptide sequence, which was removed from the electrode surface upon cleavage by PSA. Later, Deng et al. (2013), reported other peptides as specific receptors for electrochemical PSA detection; transduction was performed using hexacyanoferrate in solution. These sensing approaches were *signal-off*, which means that PSA detection was transduced into a decrease in redox current. Several other reports were very recently published, in 2015. Xie et al. (2015), reported a *signal-on* peptide cleavage-based assay to directly transduce the peptide cleavage by

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PSA into an electrochemical signal via host–guest interaction between ferrocene (Fc) and β-cyclodextrin (β-CD). Hun et al. (2015), used a self-assembled biotinylated peptide biotin-EHSSKLQKC as molecular recognition element for PSA detection, along with streptavidin-coated magnetic beads (strep-MBs) modified with the enzyme invertase. Upon cleavage, invertase is liberated and dosed using a personal glucosemeter. The detection limit is 30 pg mL^{-1}. At last, He et al. (2015), used multiwall carbon nanotubes/poly(amidoamine) dendrimers (MWCNTs–PAMAM) nanohybrides to bind the peptide probe, plus a silver stripping methodology which afforded a detection limit of 0.7 pg mL^{-1}.

In this work, we report an original reagentless (no free diffusion redox probe added in solution) electrochemical sensor based on quinone and azido moieties electrografted on an electrode by the diazonium route. The peptide probe 4-pentynoyl-GGGGHSSKLQL-OH (utile sequence underlined) was coupled on the azido group through copper-catalyzed azide-alkyne cycloaddition (also called click chemistry). This versatile approach allows control of the surface densities of both electroactive molecule and peptide probe. Two types of interactions between PSA and this peptide sequence were investigated: capture of PSA by the peptide, and cleavage of the peptide by PSA.

Transduction of the first interaction was based on steric hindrance generated by PSA. This transduction is not based on a redox molecule, such as $FeCN_6^{3-/4-}$, diffusing freely in solution nor a redox label attached to the surface through a long spacer arm. On the contrary, it is based on a compact layer of redox groups (quinone) grafted close to the electrode surface and which exchanges cations (mostly Na^+ at neutral pH in PBS buffer) during their redox process (Rubin et al., 2010). When PSA is immobilized over the electroactive grafted quinone groups, it forms a layer whose compactness modulates the counter-ion flux at the quinone/electrolyte interface, therefore lowers the redox current intensity of the quinone. The presence of PSA is therefore transduced into a decrease of current, i.e. a *signal-off*. Transduction of the second type of interaction (peptide cleavage) gave more interesting results. It was achieved by functionalization of the peptide strand at its distal end by biotin, onto which streptavidin was coupled. Cleavage cleared the surface from the heavy biotin/streptavidin conjugate, therefore leaded to a current increase (*signal-on*). The working principle of this reagentless aptamer sensor is illustrated on Fig. 1, for peptide cleavage by PSA.

Compared to the existing literature, this works is the first proof-of-concept of a reagentless (no reactant added), non-enzymatic, *signal-on* (current increase), direct (on-step) electrochemical sensor for active PSA detection.

2. Material and methods

2.1. Chemicals and reagents

Phosphate buffer saline (PBS, 0.137 M NaCl; 0.0027 M KCl; 0.0081 M Na_2HPO_4; 0.00147 M KH_2PO_4), extra dry acetonitrile (ACN), 5-hydroxy-1,4-naphthoquinone (juglone - JUG), sodium ascorbate, copper(I) sulfate pentahydrate, 4-azidoaniline (*AZA*), *t*-butyl nitrite, ethynylferrocene, tetra-*n*-butylammonium tetrafluoroborate ($TBABF_4$) and bovine serum albumin (BSA) were purchased from Sigma Aldrich. Peptide materials (sequences given in Table 1) were supplied by Eurogentec (Belgium). Aqueous solutions were made with ultrapure (18 MΩ cm) water. PSA (purity > 95%, SDS-PAGE) was provided by Meridian Life Science, Inc (Memphis, TN). Prostate Specific Antigen (f-PSA) Human ELISA Kit (ab108656) was provided by Abcam (Cambridge, UK). Streptavidin and Streptavidin-HRP conjugate were purchased from Calbiochem - Merck Millipore. Alumina slurry was from ESCIL, Chassieu, France. Glassy carbon (GC) working electrodes (3 mm in diameter, $S = 0.07\ cm^2$) were purchased from BASInc.

2.2. Methods

A three-electrode cell was used, consisting of a GC working electrode (3 mm in diameter), a Pt foil counter electrode and a commercial calomel reference electrode (SCE). Working electrodes were polished before use with 1 μm alumina slurry on a Struers Labopol-2 apparatus and rinsed with deionized water under ultrasonication during 5 min, then dried and washed with ACN. All electrochemical experiments were thermostated at 25 °C with a Peltier device (Eppendorf Thermomixer Comfort). Cyclic voltammetry (CV) and square wave voltammetry (SWV) were performed on a BioLogic VMP3 potentiostat driven with EC-Lab software. SWVs were obtained with the following parameters: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz.

Electrografting of the diazonium derivatives were performed as follow. A solution of acetonitrile (ACN) containing 30 mM *t*-butyl nitrite + 0.1 M $TBABF_4$ + 1 mM 2-[(4-aminophenyl)sulfanyl]-8-hydroxy-1,4-naphthoquinone (*Jug-φ-NH_2*) or 4-azidoaniline (*AZA*) was prepared and put in the electrochemical cell. The potential of the working GC electrode was swept between +0.35 V and −0.6 V vs. SCE at 100 mV s^{-1}. The resulting modified electrodes were washed with ACN under ultrasonication during 2 min to remove residual monomers.

Coupling of the peptide probe onto the azido group immobilized on the electrode was performed using copper-catalyzed

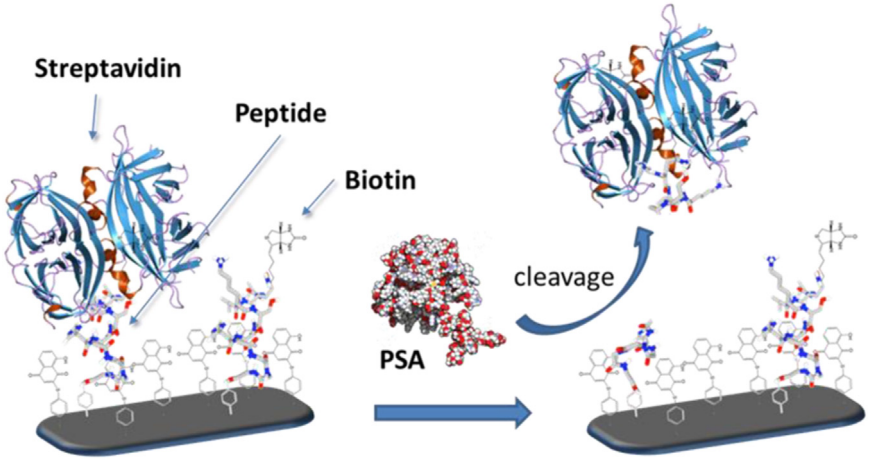

Fig. 1. Working principle of the peptide-based PSA sensor.